MB Research Labs

Study Title

Human Cell Line Activation Test (h-CLAT)

Test Article

JA900-DAA (Lot PT-917-59)

Author

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Study Completed On

23 Feb 2017

Performing Laboratory

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MB Research Project No.

MB 16-24502.41

MB Research Protocol No.

705

Sponsor

International Flavors & Fragrances, Inc.
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Union Beach, NJ 07735

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KEY PERSONNEL

George L. DeGeorge, Ph.D., DABT	Chief Scientific Officer
Micheal R. Carathers, B.S., DABT	Study Director
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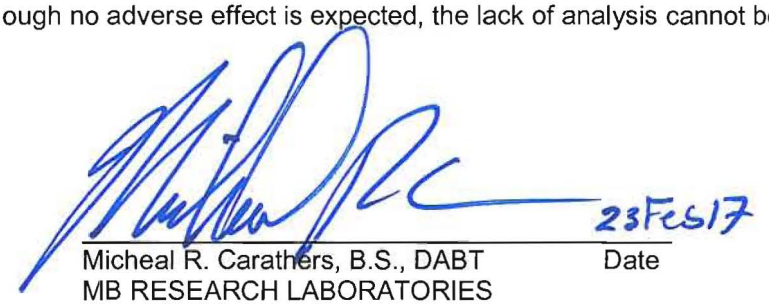
GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT

This study was conducted in accordance with the Good Laboratory Practice requirements of EPA, 40 CFR 160 and 792, FDA 21 CFR 58, and as specified in Principles on Good Laboratory Practices, published by the Organization for Economic Cooperation & Development (OECD), with the following exceptions:

Test article characterization information, provided by the Sponsor, was not conducted according to the Good Laboratory Practices. This is not expected to have an impact on the outcome of the study.

Analysis of the test article and control articles in mixtures was not performed. The mixtures were prepared fresh daily. Although no adverse effect is expected, the lack of analysis cannot be fully assessed.

STUDY DIRECTOR :



23Feb17

Micheal R. Carathers, B.S., DABT Date
MB RESEARCH LABORATORIES

QUALITY ASSURANCE EVALUATION

The Quality Assurance Unit has inspected a critical phase of this study, audited the raw data and the report and determined that the methods and results contained herein accurately reflect the raw data. A summary of the compliance inspections is presented below.

Date of Inspection	Phase	Performed By	Date Inspection Results Reported	
			Study Director	Management
27 Jul 2016	Dose Administration	Mark Coker	27 Jul 2016	27 Jul 2016
23 Sep, 27 Sep and 28 Sep 2016	Raw data audit	Cynthia M. Kelsch	28 Sep 2016	18 Nov 2016
13-14 Dec 2016	Draft report audit	Cynthia M. Kelsch	14 Dec 2016	22 Feb 2017
22 Feb 2017	Final report audit	Cynthia M. Kelsch	22 Feb 2017	22 Feb 2017

22 Feb 2017

Cynthia M. Kelsch, RQAP-GLP Date
Quality Assurance Unit

PROJECT NUMBER : MB 16-24502.41
TEST ARTICLE : JA900-DAA (Lot PT-917-59)
SPONSOR : INTERNATIONAL FLAVORS & FRAGRANCES, INC.
TITLE : HUMAN CELL LINE ACTIVATION TEST (H-CLAT)
PROTOCOL No. : 705

ABSTRACT

Objective: To determine the skin sensitization potential of a test article as assessed by the measurement of surface markers (CD86, CD54) via flow cytometry. The h-CLAT is designed to detect sensitization induced by a test article in an *in vitro* sensitization assay using the THP-1 human monocytic cell line as the test system. The assay is based on research described in Takenouchi, et al. (2013), the EURL ECVAM DataBase service on Alternative Methods to animal experimentation (DB-ALM) Protocol No. 158, and the Draft OECD Guideline "*In Vitro* Skin Sensitisation: Human Cell Line Activation Test (h-CLAT)".

Method Synopsis: Five controls were included in the study. The two positive controls were 1-chloro-2,4-dinitrobenzene (DNCB, 4 µg/ml in dimethyl sulfoxide [DMSO]) and nickel sulfate ($NiSO_4$, 100 µg/ml in 0.9% sodium chloride [saline]). The negative control was lactic acid (LA, 1000 µg/ml in saline), and the DNCB vehicle control was DMSO (0.2% in RPMI-10 medium). The RPMI-10 medium alone (100%) was also included as a control. Saline was chosen as the vehicle by the Study Director, in consultation with the Sponsor, based on solubility testing performed in a previous study (MB project no. 15-23779.41), which used the same test article.

The assay was first conducted using only the controls, not the test article, to check the reactivity of the cells. THP-1 human monocytic cells were seeded in 24-well plates at a concentration of approximately 1 x 10^6 cells in 0.5 ml of cell culture medium. Cells were dosed at one well per control and incubated for approximately 24 hours. The cells were then treated with propidium iodide (PI) plus antibody stain (for CD86 or CD54) to determine viability and induction of sensitization.

Two independent viability screens were then conducted using the test article, but not controls. THP-1 cells were seeded at approximately 1 x 10^6 cells in 0.5 ml of culture medium and dosed at one well per concentration of the test article, and incubated for approximately 24 hours, then stained with PI. Eight concentrations of the test article were tested. None of the concentrations tested produced a cell viability of less than 97.1%. Therefore, the test article concentration at which cell viability was reduced to 75% (CV75) could not be calculated.

Four main (definitive) tests, as independent assays, were then conducted in the same manner as the screen, but with both test article and controls, to determine CD86 and CD54 expression. The test article was assayed at eight concentrations, using a maximum concentration of 5000 µg/ml in saline, with 1.2-fold serial dilutions. Main Test 2 failed to pass the quality control acceptance criteria for the DNCB positive control and the DMSO vehicle control; the other three main tests passed all acceptance criteria. The effective concentration (EC) values (i.e., the concentration at which the test article induced a CD54 RFI of 200 or a CD86 RFI value of 150) could not be calculated.

Conclusion: Test article JA900-DAA (Lot PT-917-59) produced a negative response in both CD54 and CD86 in THP-1 human monocytic cells in two of three valid independent main tests conducted. Therefore, this test article is not considered a potential dermal sensitizer in the Human Cell Line Activation Test (h-CLAT). The EC150 and EC200 values could not be calculated because no treatment in the two negative main tests produced an RFI values greater than the cutoff for a positive response. Also, the positive response (Main Test 1) showed an up-and-down response in both CD86 and CD54; therefore no clear EC150 or EC200 values could be calculated.

OBJECTIVE

To determine the skin sensitization potential of a test article as assessed by the measurement of surface markers (CD86, CD54) via flow cytometry. The h-CLAT is designed to detect sensitization induced by a test article in an *in vitro* sensitization assay using the THP-1 human monocytic cell line as the test system. The assay is based on research described in Takenouchi, et al. (2013), the EURL ECVAM DataBase service on Alternative Methods to animal experimentation (DB-ALM) Protocol No. 158, and the Draft OECD Guideline "*In Vitro* Skin Sensitisation: Human Cell Line Activation Test (h-CLAT)".

TEST ARTICLE

Identity	:	JA900-DAA (Lot PT-917-59)
Provided by	:	International Flavors & Fragrances, Inc.
Test Article Characterization	:	See Appendix A for Test Article Characterization
Date Received	:	23 Jun 2016
Storage	:	Room temperature and humidity, protected from light
Description	:	Clear colorless liquid
Sample Preparation	:	The formulations were freshly prepared prior to use. Preparation and dosing were conducted under yellow lights, to protect the formulations from fluorescent light.

Stock Formulations:
Screens – 500 mg of the test article were brought to a total volume of 1 ml with saline to yield a 500,000 µg/ml formulation. Seven additional formulations (250,000 µg/ml, 125,000 µg/ml, 62,500 µg/ml, 31,250 µg/ml, 15,625 µg/ml, 7,812.5 µg/ml, and 3,906.3 µg/ml) were made with saline.

Main Tests 1, 2 and 3 – 500 mg of the test article were brought to a total volume of 1 ml with saline to yield a 500,000 µg/ml formulation. Seven additional formulations (416,670 µg/ml,345,840 µg/ml, 287,500 µg/ml, 239,590 µg/ml, 200,000 µg/ml, 166,670 µg/ml, and 139,580 µg/ml) were made with saline.

Main Test 4 – 500 mg of the test article were brought to a total volume of 1 ml with saline to yield a 500,000 µg/ml formulation. Seven additional formulations (416,500 µg/ml,346,944.5 µg/ml, 289,004.8 µg/ml, 240,741 µg/ml, 200,537.3 µg/ml, 167,047.6 µg/ml, and 139,150.7 µg/ml) were made with saline.

Note: Dose concentrations were listed slightly differently in Main Test 4 as compared to Main Tests 1, 2, and 3. Main Test 4 dose concentrations were calculated using the actual volumes used for each stock solution, which was then further diluted in media. Dose concentrations in Main Tests 1, 2 and 3 were back-calculated from dose concentrations computed by MS Excel®. However, all of the main tests were diluted and dosed in the same manner.

TEST ARTICLE (continued)

Sample Preparation (continued):

Working Formulations:
Screens – For each test article stock formulation, 50 µl of the stock were added to 2,450 µl of culture media (diluting the stock by 50 times) to yield working formulations of 10,000 µg/ml, 5,000 µg/ml, 2,500 µg/ml, 1,250 µg/ml, 625 µg/ml, 312.5 µg/ml, 156.3 µg/ml, and 78.1 µg/ml. Final dose concentrations were 5,000 µg/ml, 2,500 µg/ml, 1,250 µg/ml, 625 µg/ml, 312.5 µg/ml, 156.3 µg/ml, 78.2 µg/ml, and 39.1 µg/ml.

Main Tests 1, 2 and 3 – For each test article stock formulation, 50 µl of the stock were added to 2,450 µl of culture media (diluting the stock by 50 times) to yield working formulations of 10,000 µg/ml, 8,333.4 µg/ml, 6,916.8 µg/ml, 5,750 µg/ml, 4,791.8 µg/ml, 4,000 µg/ml, 3,333.4 µg/ml, and 2,791.6 µg/ml. Final dose concentrations were 5,000 µg/ml, 4,166.7 µg/ml, 3,458.4 µg/ml, 2,875.0 µg/ml, 2,395.9 µg/ml, 2,000 µg/ml, 1,666.7 µg/ml, and 1,395.8 µg/ml.

Main Test 4 – For each test article stock formulation, 50 µl of the stock were added to 2,450 µl of culture media (diluting the stock by 50 times) to yield working formulations of 10,000 µg/ml, 8,330 µg/ml, 6,938.9 µg/ml, 5,780.1 µg/ml, 4,814.8 µg/ml, 4,010.7 µg/ml, 3,341 µg/ml, and 2,783 µg/ml. Final dose concentrations were 5,000 µg/ml, 4,165 µg/ml, 3,469.5 µg/ml, 2,890.1 µg/ml, 2,407.4 µg/ml, 2,005.4 µg/ml, 1,670.5 µg/ml, and 1,391.5 µg/ml.

POSITIVE CONTROLS

Identity	:	1-Chloro-2,4-dinitrobenzene (DNCB), Lot No. STBF4847V (See Appendix B for Certificate of Analysis)
Supplied by	:	Aldrich
Date Received	:	27 Feb 2016
Expiration Date	:	27 Feb 2017
Storage	:	Room temperature and humidity.
Description	:	Yellow crystals
Sample Preparation	:	The solutions were freshly prepared prior to use. Preparation and dosing were conducted under yellow lights, to protect the solutions from fluorescent light. Stock Solution: 20 mg of DNCB were brought to a total volume of 10 ml with DMSO to yield a 2 mg/ml stock solution. Working Solution: 10 µl of DNCB stock solution were added to 2,490 µl of culture media (diluting the stock by 250 times) to yield an 8 µg/ml working solution. The final dose concentration was 4 µg/ml.

Identity	:	Nickel Sulfate ($NiSO_4$), Lot No. A0357363 (See Appendix B for Certificate of Analysis)
Supplied by	:	Acros Organics
Date Received	:	16 Dec 2015
Expiration Date	:	28 Dec 2016
Suggested Retest Date	:	Mar 2020
Storage	:	Room temperature and humidity.
Description	:	Blue powder
Sample Preparation	:	The solutions were freshly prepared prior to use. Preparation and dosing were conducted under yellow lights, to protect the solutions from fluorescent light. Stock Solution: 100 mg of $NiSO_4$ were brought to a total volume of 10,000 µl with saline to yield a 10 mg/ml stock solution. Working Solution: 50 µl of $NiSO_4$ stock solution were added to 2,450 µl of culture media (diluting the stock by 50 times) to yield a 200 µg/ml working solution. The final dose concentration was 100 µg/ml.

VEHICLE CONTROL FOR THE DNCB POSITIVE CONTROL

Identity	:	0.2% dimethylsulfoxide (DMSO) in RPMI-10 medium
Prepared by	:	MB Research
Dates Prepared	:	Reactivity Check: 11 Jul 2016 Main Tests: 27 Jul 2016, 01 Aug 2016, 03 Aug 2016, and 08 Aug 2016
Sample Preparation	:	The solution was freshly prepared prior to use. Preparation and dosing were conducted under yellow lights, to protect the solution from fluorescent light. 10 µl of DMSO were added to 2,490 µl of culture medium to yield a 0.4% solution. The final dose concentration was 0.2%.

NEGATIVE CONTROL

Identity	:	Lactic Acid (LA), Lot No. BCBM8154V (See Appendix B for Certificate of Analysis)
Supplied by	:	Fluka
Date Received	:	25 Nov 2014
Expiration Date	:	Feb 2018
Storage	:	Room temperature and humidity.
Description	:	Clear colorless liquid
Sample Preparation	:	The solutions were freshly prepared prior to use. Preparation and dosing were conducted under yellow lights, to protect the solutions from fluorescent light. Stock Solution: 100 mg of LA were brought to a total volume of 1,000 µl with saline to yield a 100 mg/ml stock solution. Working Solution: 50 µl of LA stock solution was added to 2,450 µl of culture medium (diluting the stock by 50 times) to yield a 2,000 µg/ml working solution. The final dose concentration was 1,000 µg/ml.

VEHICLES

Identity	:	Dimethylsulfoxide (DMSO), Lot No. 161028 (vehicle for DNCB) (See Appendix B for Certificate of Analysis)
Supplied by	:	Fisher Scientific
Date Received	:	29 Apr 2016
Expiration Date	:	Mar 2021
Storage	:	Room temperature and humidity.
Description	:	Clear colorless liquid
Sample Preparation	:	Used as received

Identity	:	0.9% Sodium Chloride (saline), Lot No. 38-603-4B-02 (vehicle for the test article, $NiSO_4$ and LA) (See Appendix B for Certificate of Analysis)
Supplied by	:	Hospira
Date Received	:	06 Jun 2014
Expiration Date	:	01 Feb 2017
Storage	:	Room temperature and humidity.
Description	:	Clear colorless liquid
Sample Preparation	:	Used as received

MEDIUM

Identity	:	RPMI-1640 culture medium supplemented with 1% penicillin-streptomycin, 0.05 mM 2-mercaptoethanol solution, and 10% fetal bovine serum (RPMI-10 medium)
Prepared By	:	MB Research
Dates Prepared	:	10 Jun 2016, 08 Jul 2016, 15 Jul 2016, 22 Jul 2016, 27 Jul 2016, and 03 Aug 2016
Expiration Dates	:	10 Jul 2016, 08 Aug 2016, 15 Aug 2016, 22 Aug 2016, 27 Aug 2016, and 03 Sep 2016
Storage	:	Refrigerated at 2-8°C
Description	:	Clear red liquid
Sample Preparation	:	0.176 ml of a 0.142 M 2-mercaptoethanol solution, 12.5 ml of 1 M HEPES buffer solution, 5 ml of penicillin-streptomycin and 50 ml Fetal Bovine Serum were added to 432.5 ml of RPMI-1640 medium and filter-sterilized.

COMPONENTS OF THE MEDIUM

Identity	:	RPMI-1640 culture medium, Lot No. AAK205984 and ABB213926 (See Appendix B for Certificates of Analysis)
Supplied by	:	Hyclone™
Dates Received	:	29 Apr 2016 and 07 Jan 2016; 02 Aug 2016
Expiration Dates	:	Oct 2016; Feb 2017
Storage	:	Refrigerated at 2-8°C
Description	:	Clear red liquid
Sample Preparation	:	Used as received
Identity	:	1 M HEPES buffer solution, Lot No. BCBR0190V (See Appendix B for Certificate of Analysis)
Supplied by	:	Sigma
Date Received	:	06 Jul 2016
Retest Date	:	Apr 2017
Storage	:	Room temperature and humidity
Description	:	Clear colorless liquid
Sample Preparation	:	Used as received
Identity	:	Penicillin-Streptomycin, Lot No. 159063 (See Appendix B for Certificate of Analysis)
Supplied by	:	Fisher Scientific
Date Received	:	17 Jun 2016
Expiration Date	:	Aug 2017
Storage	:	Refrigerated at 2-8°C
Description	:	Clear colorless liquid
Sample Preparation	:	Used as received
Identity	:	2-Mercaptoethanol, Lot No. QC215754A (See Appendix B for Certificate of Analysis)
Supplied by	:	ThermoFisher Scientific
Date Received	:	06 Apr 2015
Expiration Date	:	30 Mar 2017
Storage	:	Refrigerated at 2-8°C
Description	:	Clear colorless liquid
Sample Preparation	:	The 14.2 M 2-mercaptoethanol solution was diluted 1:99 with tissue culture water to yield a 0.142 M 2-mercaptoethanol solution.

COMPONENTS OF THE MEDIUM (continued)

Identity	:	Tissue Culture Water (TCH_2O), Lot No. RNBF0858 (See Appendix B for Certificate of Analysis)
Supplied by	:	Sigma
Date Received	:	22 Mar 2016
Expiration Date	:	Nov 2017
Storage	:	Room temperature and humidity
Description	:	Clear colorless liquid
Sample Preparation	:	Used as received

Identity	:	Fetal Bovine Serum, Lot No. FBU15678HI and FBU15680HI (See Appendix B for Certificates of Analysis)
Supplied by	:	Serum Source International
Date Received	:	04 Feb 2016 and 12 Apr 2016; 21 Jun 2016
Expiration Date	:	Sep 2019 and Nov 2020
Storage	:	Refrigerated at 2-8°C
Description	:	Clear brown liquid
Sample Preparation	:	Used as received

TEST SYSTEM

Identity : THP-1 cells: Acute Monocytic Leukemia, Human, ATCC No.TIB-202

Supplied by : American Type Culture Collection (ATCC)

Lot Number : 60731979

Date Received : 21 Feb 2014

Media : RPMI-1640 culture medium supplemented with 1% penicillin-streptomycin, 0.05 mM 2-mercaptoethanol solution, and 10% fetal bovine serum (RPMI-10 medium)

Passage :

Phase	Test No.	Dose Date	Passage
Reactivity Check	1	11 Jul 2016	14
Screen	1	13 Jul 2016	15
	2	20 Jul 2016	18
Main	1	27 Jul 2016	21
	2	01 Aug 2016	23
	3	03 Aug 2016	24
	4	08 Aug 2016	26

TEST DATES

Study Initiation	(date protocol signed)	:	07 Jul 2016
Experimental Start Date	(1st date data collected – OECD)	:	11 Jul 2016
Experimental Start Date	(1st exposure to test substance)	:	13 Jul 2016
Experimental Term Date	(last date data collected)	:	09 Aug 2016
Draft Report Submitted	(if applicable)	:	22 Dec 2016
Final Report Signed	(study completion)	:	23 Feb 2017

EXPERIMENTAL DESIGN

Basis of the Method

Cell viability was obtained for each test article concentration by PI staining and flow cytometric analysis. For prediction of cytotoxicity and sensitization potential, the concentration responses obtained in the presence of test article were compared, usually at the CV75 level, i.e., the concentration at which cell viability is approximately 75%. Any increases in CD86 and CD54 markers above vehicle control levels was assessed to determine if the test article had sensitization potential around the CV75 dose levels.

The test contained three parts:

1. **Reactivity check** to ensure that the cells were growing adequately and performing properly. The assay was first conducted using only controls, not the test article, to check the reactivity of the cells. The reactivity check was conducted once.

2. **Viability screens** to determine the CV75 value. The screen was conducted twice, in independent assays. Eight concentrations of the test article were tested, to a maximum concentration of 5,000 µg/ml. None of the concentrations tested produced a cell viability of less than 97.1%. Therefore, the test article concentration at which cell viability was reduced to 75% (CV75) could not be calculated.

3. **Main tests** to determine CD86 and CD54 expression. Since the CV75 could not be determined, eight concentrations of the test article were tested using a maximum concentration of 5000 µg/ml (as per the guideline) in saline, with 1.2-fold serial dilutions. The main test was conducted four times, in independent assays.

Controls

Five controls were included in the study. The two positive controls were DNCB (4 µg/ml in DMSO) and $NiSO_4$ (100 µg/ml in saline). The negative control was lactic acid (100 µg/ml in saline), and the vehicle control was DMSO (0.2% in RPMI-10 medium). The RPMI-10 medium alone (100%) was also included as a control.

EXPERIMENTAL DESIGN (continued)

Pre-test Preparation of THP-1 Cells

The immortalized human monocytic leukemia cell line THP-1 was used as a surrogate for human dendritic cells. An aliquot of the cell stock was thawed according to the cell bank's instructions, added to fresh culture medium, and incubated at 37±1ºC, 5±1% CO_2. Cells were maintained in suspension at densities from approximately 1 x 10^5 to 8 x 10^5 cells/ml. Cells were routinely passaged every two to three days at a seeding density of approximately 2 x 10^5 cells/ml.

Reactivity Check

The assay was first conducted using only the control articles, not the test article, to ensure that the cells were adequately performing.

Dosing:

The THP-1 cell line was plated and grown in T75 culture flasks (37°C ± 1ºC, 5% ±1% CO_2) in growth medium to obtain a sufficient quantity of cells for the experiment. Cells were centrifuged (approximately 250g for approximately 5 minutes at 2-8ºC) and re-suspended in fresh culture medium at a density of approximately 2x10^6 cells/ml. Using a pipette, 500 µl of cell suspension were dispensed into the wells of a 24-well tissue culture plate. 500 µl of the working solution for each control were added to the cell suspension in the appropriate well. The final concentrations of each control were 4 µg/ml for DNCB, 100 µg/ml for $NiSO_4$, and 1000 µg/ml for LA. The cells were then incubated for approximately 24 hours (37±1ºC, 5±1% CO_2).

Cell Staining:

Following the 24-hour incubation, the cells were transferred from each well to 1.5 ml Eppendorf tubes. The tubes were centrifuged and the cells were washed twice in FACS buffer, then re-suspended in blocking solution and incubated at 2-8°C for approximately 15 minutes. Aliquots of the cell supernatant (approximately 3 x 10^5 cells/well) were transferred into three wells of a round-bottom plate, centrifuged, and the supernatant was aspirated. Cells were then stained for CD86, CD54, or isotype control antibodies and incubated at 2-8°C for approximately 30 minutes. Isotype is an IgG antibody tagged with fluorescein isothiocyanate (FITC) that is used to determine background antibody staining (non-specific). Following incubation, the stained cells were centrifuged, washed twice with FACS buffer, and re-suspended in FACS buffer and transferred to flow cytometry tubes. 10 µl of PI solution was added to each flow cytometry tube to obtain a final PI concentration of 0.658 µg/ml and the tubes were analyzed by flow cytometry.

Flow Cytometry:

Flow cytometric analyses were conducted using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an Omnichrome argon laser emitting at 488 nm with 15 mW of power. Clumps of nuclei were excluded from analysis using gates set on integrated red fluorescence signals. BD CellQuest version 3.3 acquisition software (Becton Dickinson Immunocytometry Systems, San Jose, CA) on a Macintosh G4 acquisition system was used to capture and store data on a dedicated secure network drive.

EXPERIMENTAL DESIGN (continued)

Reactivity Check (continued)

Cell Viability:

Cell viability was measured by flow cytometry, gating out dead cells stained with PI. A total of approximately 10,000 living cells were acquired. Viability was calculated as a percent of total cells by the following equation:

$$\text{Cell Viability} = \frac{\text{Number of living cells}}{\text{Total number of acquired cells}} \text{ X } 100$$

For each treatment, viability was measured for isotype, CD86 and CD54, and the mean viability was then calculated.

Reactivity Check Acceptance:

The reactivity check is considered acceptable if:

- The viability of non-treated cells cultured in the culture medium for 24 hours was more than 90%
- Treatment of the cells with DNCB and $NiSO_4$ produced a positive response for both CD86 (RFI greater than or equal to 150) and CD54 (RFI greater than or equal to 200)
- Treatment of the cells with LA produced a negative response for both CD86 and CD54

EXPERIMENTAL DESIGN (continued)

Viability Screens

Two independent viability screens were conducted, using only the test article, not controls. Cell viability was measured by flow cytometry.

Dosing:

Cell suspensions were prepared in the same manner as in the reactivity check. Using a pipette, 500 µl of cell suspension were dispensed into the wells of a 24-well tissue culture plate. 500 µl of the working solution for each test article concentration were added to the cell suspension in the appropriate well. The cells were incubated for approximately 24 hours (37±1ºC, 5±1% CO_2).

Cell Staining:

Following the 24-hour incubation, the cells were transferred from each well to 1.5 ml Eppendorf tubes. The tubes were centrifuged, the supernatant was aspirated, and the cells were re-suspended in FACS buffer. Aliquots of the cell suspension were transferred into the wells of a round-bottom or V-bottom plate. The cells were centrifuged, washed twice with FACS buffer, and then re-suspended in FACS buffer. 10 µl of PI were added to the cell suspensions to obtain a final PI concentration of 0.625 µg/ml, which were then transferred to flow cytometry tubes and analyzed by flow cytometry.

Cell Viability:

Cell viability was measured by flow cytometry, gating out dead cells stained with PI. A total of approximately 10,000 living cells were acquired. Viability was calculated as a percent of total cells by the following equation:

$$\text{Cell Viability} = \frac{\text{Number of living Cells}}{\text{Total number of acquired cells}} \text{ X } 100$$

Viability was measured once for each test article concentration for each screen.

EXPERIMENTAL DESIGN (continued)

Estimation of CV75 Value

Since none of the concentrations tested in the screens produced a cell viability of less than 97.1%, and the maximum concentration tested could not be increased due to the limit set by the guideline, the CV75 value could not be calculated.

Main Tests

Four independent main tests were conducted, using both the test articles and the controls. Dosing and cell staining was performed in the same manner as in the reactivity checks, except that the PI concentration was 0.625 µg/ml. For each treatment, flow cytometry analysis was used to measure cell viability and the Mean Fluorescence Intensity (MFI) of the viable cells for isotype, CD86, and CD54 (see Analysis of Data).

The second main test failed to meet the quality control acceptance criteria for the DNCB positive control and the DMSO vehicle control, so the main test was repeated. Since the results of the third main test were not consistent with those of the first main test, a fourth main test was conducted.

Analysis of Data

The Geometric Mean (GeoMean) Fluorescence Intensity (MFI) for each well was measured by flow cytometry and corrected for background by subtracting the isotype value. As an indicator of CD86 and CD54 expression, the Relative Fluorescence Intensity (RFI) was then calculated for each test article concentration and control using the following equation:

$$\text{RFI} = \frac{\text{MFI of chemical-treated cells} - \text{MFI of chemical-treated isotype cells}}{\text{MFI of solvent-treated cells} - \text{MFI of solvent-treated isotype cells}}$$

Treatment with the test article in Main Test 1 produced positive responses for both CD86 and CD54, and the calculated RFI values were inconsistent and not dose-dependent.

Main Test 2 was invalid due to failure to pass the quality control acceptance criteria. Main Tests 3 and 4 were valid tests.

The EC150 and EC200 values could not be calculated because no treatment in the two negative main tests produced an RFI values greater than the cutoff for a positive response. Also, the positive response (Main Test 1) showed an up-and-down response in both CD86 and CD54; therefore no clear EC150 or EC200 values could be calculated.

Interpretation of the Data

If the RFI of CD86 is equal to or greater than 150 at any test dose (cell viability of more than 50%) in at least two independent assays, and/or if the RFI of CD54 is equal to or greater than 200 at any tested dose (cell viability of more than 50%) in at least two independent assays, the chemical prediction was considered positive. Otherwise it was considered negative.

EXPERIMENTAL DESIGN (continued)

Quality Checks of the Assay (Main Test)

Test Article:

The cell viability of at least four test article concentrations in each assay should be 50% or more. Negative results are acceptable only for test chemicals exhibiting cell viability at 1.2x CV75 of less than 90%. Negative results with cell viability of 90% or higher are discarded. The dose finding study should be retested to determine the CV75 determination. Positive results for test chemicals of any cell viability at 1.2x CV75 are acceptable. It should be noted that when 5000 µg/ml in saline, 1000 µg/ml in DMSO, or the highest soluble concentration is used as the maximal test concentration of a test chemical, the results are acceptable.

Positive Controls:

DNCB and $NiSO_4$ positive controls should each produce a positive response for both CD86 (RFI of 150 or more) and CD54 (RFI of 200 or more) as compared to the negative control. In addition, cell viability for each positive control should be greater than 50%.

Negative Controls:

DMSO and lactic acid RFI values compared to medium control for both CD86 and CD54 should not exceed the positive criteria (CD86 greater than or equal to 150 and CD54 greater than or equal to 200). For both medium and DMSO controls, the MFI ratio for both CD86 and CD54 to isotype control should be greater than 105%. In addition, cell viability of medium and DMSO controls should be greater than 90%.

Retention of the Data

Upon signing the final report, all raw data supporting documentation and reports are submitted to the Archivist by the Study Director. The raw data are filed at MB Research by project number. The final report is filed at MB Research by Sponsor name and MB project number.

All data generated during the conduct of this study are archived at MB Research for at least ten years from the date of the final report. The Sponsor will be contacted in writing to determine final disposition of the records. If the Sponsor fails to respond within 90 days, the archived items will be properly discarded. Any remaining test article will be discarded upon submission of the report.

Amendment to the Protocol

See Appendix C for the protocol in its entirety.

Deviation from the Protocol

In the reactivity check, the dilution of PI for antibody staining was done incorrectly, resulting in too high a concentration of PI in each tube. This is not expected to have an impact on the outcome of the study. All controls passed QC specifications. The DNCB and $NiSO_4$ positive controls both produced a positive response when compared to the DMSO control, and the lactic acid negative control produced a negative response when compared to the media control. The reactivity check met all QC specifications for a valid test.

RESULTS

Reactivity Check

The assay was first conducted using only controls, not the test article. See Table 1 for experimental data.

	Reactivity Check			
Treatment	**Mean Viability (%)**	**CD86 RFI**	**CD54 RFI**	**Pass / Fail**
Media (RPMI-10)	97.4	*NA*	*NA*	PASS
DNCB, 4 µg/ml	*NA*	411	592	PASS
$NiSO_4$, 100 µg/ml	*NA*	154	237	PASS
LA, 1000 µg/ml	*NA*	49	74	PASS

NA = not applicable

All acceptance criteria were met. The cells were judged to be growing adequately and performing properly, so the assay continued as per the protocol.

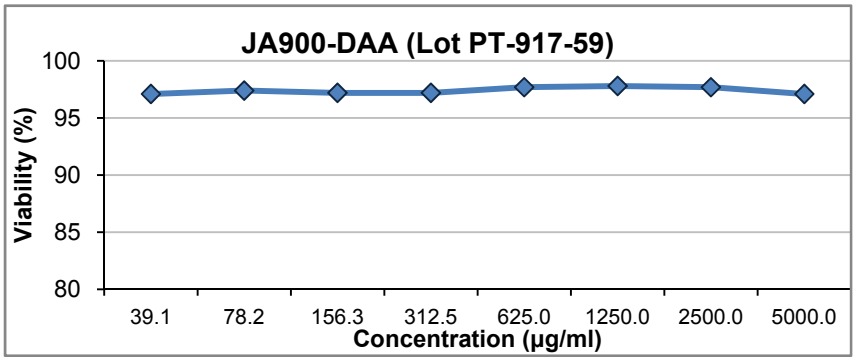
RESULTS (continued)

Viability Screens

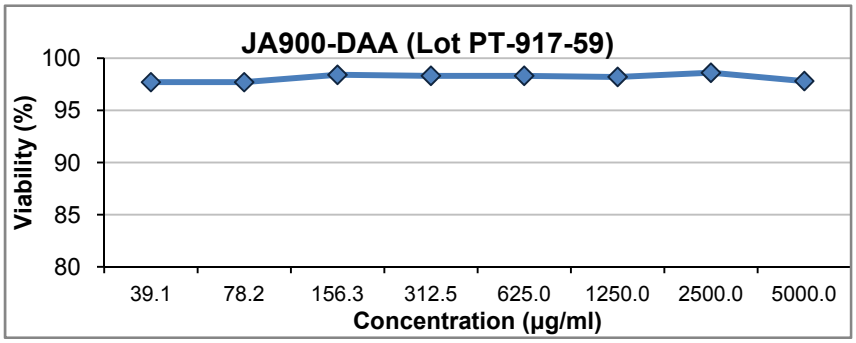
Two independent viability screens were conducted, using only the test article, not controls. Since none of the concentrations tested in the screens produced a cell viability of less than 97.1%, and the maximum concentration tested could not be increased due to the limit set by the guideline, the CV75 value could not be calculated.

See Table 2 for experimental data.

Screen 1



Screen 2



RESULTS (continued)

Main Tests - Quality Checks

Test Article:

The mean cell viabilities for all eight concentrations of the test article in the main tests were as follows:

	Mean Viability Range (%)
Main Test 1	96.5 – 97.8
Main Test 2	95.5 – 96.7
Main Test 3	95.3 – 96.8
Main Test 4	94.0 – 95.8

All tests passed the acceptance criterion of a mean cell viability of 50% or more for at least four concentrations.

Positive Controls:

Main Tests 1, 3 and 4: For both the DNCB and the $NiSO_4$ positive controls, the mean cell viabilities were greater than 50%, the CD86 RFI values were greater than 150, and the CD54 RFI values were greater than 200. The positive controls passed all acceptance criteria (RFI of 150 or more for CD86, or RFI of 200 or more CD54) in each of these main tests.

Main Test 2: The DNCB positive control mean cell viability was less than 50%, which failed the acceptance criteria of greater than 50%. Also, the CD86 RFI was less than 150, and the CD54 RFI was less than 200, which failed the acceptance criteria.

The $NiSO_4$ positive control had a mean viability greater than 50%, a CD86 RFI value greater than 150 and a CD54 RFI value greater than 200, which passed the acceptance criteria.

Due to failure of the DNCB positive control, Main Test 2 was considered invalid.

Positive Control	DNCB			$NiSO_4$		
	Mean Viability (%)	CD86 RFI	CD54 RFI	Mean Viability (%)	CD86 RFI	CD54 RFI
Main Test 1	96.0	375 ✶	392 ✶	93.8	564 ✶	397 ✶
Main Test 2	41.9[1]	125 [2]	107 [2]	93.7	231 ✶	296 ✶
Main Test 3	51.3	202 ✶	1071 ✶	94.1	224 ✶	1190 ✶
Main Test 4	53.0	355 ✶	898 ✶	86.9	205 ✶	498 ✶

✶ = positive sensitizing response (RFI of 150 or more for CD86 or RFI of 200 or more for CD54)
1 = failed to meet the acceptance criterion of more than 50%
2 = failed to meet the acceptance criterion of RFI of 150 or more for CD86 or RFI of 200 or more for CD54

RESULTS (continued)

Main Tests - Quality Checks (continued)

Negative Controls:

Main Tests 1, 3 and 4: The mean cell viabilities for the DMSO and lactic acid negative controls, and for the media control, were all greater than 90%. For the DMSO negative control, the CD86 RFI values were less than 150, and the CD54 RFI values were less than 200, indicating a negative sensitization response. For both medium and DMSO controls, the MFI ratios of both CD86 and CD54 to isotype control were greater than 105%. The negative controls passed all acceptance criteria in each of these main tests.

Main Test 2: The mean cell viabilities for the DMSO and lactic acid negative controls, and for the media control, were all greater than 90%, which passed the acceptance criteria. The DMSO negative control, CD86 RFI value was greater than 150, which failed the acceptance criteria (RFI less than 150); the CD54 RFI value was less than 200, which passed the acceptance criteria. The media control and the lactic acid negative control passed all acceptance criteria.

Due to failure of the DMSO negative control, Main Test 2 was considered invalid.

			RFI		MFI			MFI Ratio	
	Negative Control	Mean Viability (%)	CD86 vs. Medium	CD54 vs. Medium	Isotype	CD86	CD54	CD86 vs. Isotype	CD54 vs. Isotype
Main Test 1	Medium	97.2	*NA*	*NA*	2.27	2.63	2.66	115.9%	117.2%
	DMSO	97.4	142	67	2.64	3.15	2.90	119.3%	109.8%
	Lactic Acid	96.8	*NA*	*NA*	2.15	2.44	2.36	*NA*	*NA*
Main Test 2	Medium	96.0	*NA*	*NA*	1.97	2.84	2.54	144.2%	128.9%
	DMSO	95.9	157[1]	132	2.11	3.48	2.86	164.9%	135.5%
	Lactic Acid	95.6	*NA*	*NA*	1.61	2.36	2.17	*NA*	*NA*
Main Test 3	Medium	96.1	*NA*	*NA*	2.11	3.12	2.31	147.9%	109.5%
	DMSO	96.5	130	190	2.02	3.33	2.40	164.9%	118.8%
	Lactic Acid	94.8	*NA*	*NA*	1.75	2.40	1.96	*NA*	*NA*
Main Test 4	Medium	95.5	*NA*	*NA*	2.17	3.67	2.71	169.1%	124.9%
	DMSO	96.9	105	89	2.10	3.67	2.58	174.8%	122.9%
	Lactic Acid	96.8	*NA*	*NA*	1.86	2.65	2.21	*NA*	*NA*

NA = not applicable
1= failed to meet the acceptance criteria of less than 150

RESULTS (continued)

Main Tests – Experimental Data

Since the CV75 could not be determined, eight concentrations of the test article were tested using a maximum concentration of 5000 µg/ml in saline, with 1.2-fold serial dilutions. The main test was conducted four times, in independent assays.

Dose concentrations were listed slightly differently in Main Test 4 as compared to Main Tests 1, 2, and 3. Main Test 4 dose concentrations were calculated using the actual volumes used for each stock solution, which was then further diluted in media. Dose concentrations in Main Tests 1, 2 and 3 were back-calculated from dose concentrations computed by MS Excel®. However, all of the main tests were diluted and dosed in the same manner.

Treatment with the test article in Main Test 1 produced positive responses for both CD86 and CD54. However, the calculated RFI values were inconsistent and not dose-dependent.

Main Test 2 was invalid due to failure to pass the quality control acceptance criteria.

Main Tests 3 and 4 were valid tests. Since treatment with the test article concentrations in either main tests did not yield RFI values both above and below the positive criteria (RFI of 150 for CD86, or 200 for CD54), the effective concentration (EC) values (i.e., the concentration at which the test article induced an RFI of 150 or 200) could not be calculated and was deemed negative for sensitization.

See Table 3 for experimental data.

Main Test RFI Values

	Main Test 1		Main Test 2[1]		Main Test 3	
Concentration	RFI CD86	RFI CD54	RFI CD86	RFI CD54	RFI CD86	RFI CD54
1395.8 µg/ml	303 *	221 *	90	63	78	140
1666.7 µg/ml	108	110	83	72	87	105
2000 µg/ml	333 *	159	71	89	106	95
2395.9 µg/ml	150 *	54	80	82	65	45
2875 µg/ml	139	97	124	86	74	90
3458.4 µg/ml	264 *	77	85	109	83	135
4166.7 µg/ml	228 *	82	91	135	90	180
5000 µg/ml	206 *	131	99	135	101	155

	Main Test 4	
Concentration	RFI CD86	RFI CD54
1391.5 µg/ml	57	57
1670.5 µg/ml	61	91
2005.4 µg/ml	49	93
2407.4 µg/ml	84	115
2890.1 µg/ml	73	122
3469.5 µg/ml	75	139
4165 µg/ml	98	150
5000 µg/ml	85	119

* = positive sensitizing response (RFI of 150 or more for CD86 or RFI of 200 or more for CD54)
1 = test was invalid due to failure to pass the quality control acceptance criteria

REFERENCES

1. Takenouchi, O., Miyazawa, M., Saito, K., Ashikaga, T., and Sakaguchi, H. Predictive performance of the human Cell Line Activation Test (h-CLAT) for lipophilic chemicals with octanol-water partition coefficients. The Journal of Toxicological Sciences (J. Toxicol, Sci,) Vol 38, No.4, 599-609, 2013.

2. EURL ECVAM DataBase service on Alternative Methods to animal experimentation (DB-ALM) protocol no. 158: Human cell line activation test (h-CLAT), 2014.

3. Draft OECD Guideline "*In Vitro* Skin Sensitisation: Human Cell Line Activation Test (h-CLAT).

4. KOWWIN™, in Estimation Program Interface (EPI) suite™, Environmental Protection Agency, Washington, DC, USA (http://www2.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface)

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6. ALOGPS, Virtual Computational Chemistry Laboratory (http://vcclab.org/lab/alogps/)

CONCLUSION

Test article JA900-DAA (Lot PT-917-59) produced a negative response in both CD54 and CD86 in THP-1 human monocytic cells in two of three valid independent main tests conducted. Therefore, this test article is not considered a potential dermal sensitizer in the Human Cell Line Activation Test (h-CLAT). The EC150 and EC200 values could not be calculated because no treatment in the two negative main tests produced an RFI values greater than the cutoff for a positive response. Also, the positive response (Main Test 1) showed an up-and-down response in both CD86 and CD54; therefore no clear EC150 or EC200 values could be calculated.

FINAL REPORT

Approved by:

23Feb17

Micheal R. Carathers, B.S., DABT Date
Study Director

Table 1: Reactivity Check - Experimental Data

Treatment	Control	Isotype	CD86	CD54	CD86		CD54		Viability (%)			
		MFI	MFI	MFI	Corrected MFI	RFI	Corrected MFI	RFI	Isotype	CD86	CD54	Mean
Media	Media	2.27	4.35	3.18	2.08	100	0.91	100	97.2	97.5	97.6	97.4
DMSO, 0.2%	DMSO	2.31	4.18	3.05	1.87	100	0.74	100	96.9	97.2	97.0	97.0
DNCB, 4 µg/ml	DMSO	2.39	10.07	6.77	7.68	411 *	4.38	592 *	84.1	84.6	84.2	84.3
$NiSO_4$, 100 µg/ml	Media	2.42	5.63	4.58	3.21	154 *	2.16	237 *	92.1	97.7	93.7	94.5
LA, 1000 µg/ml	Media	2.09	3.11	2.76	1.02	49	0.67	74	96.9	97.1	97.1	97.0

* = positive sensitizing response (RFI of 150 or more for CD86, or RFI of 200 or more for CD54)

Table 2: Viability Screens - Experimental Data

Concentration (µg/ml)	Screen 1		Screen 2	
	Viability (%)	CV75 (µg/ml)	Viability (%)	CV75 (µg/ml)
39.1	97.1	Could not be calculated	97.7	Could not be calculated
78.2	97.4		97.7	
156.3	97.2		98.4	
312.5	97.2		98.3	
625.0	97.7		98.3	
1250.0	97.8		98.2	
2500.0	97.7		98.6	
5000.0	97.1		97.8	

Since none of the concentrations tested in the screens produced a cell viability of less than 97.1%, and the maximum concentration tested could not be increased due to the limit set by the guideline, the CV75 value could not be calculated.

Table 3: Main Tests - Experimental Data

Main Test 1			Isotype		CD86					CD54					Mean[1] Viability (%)
							RFI					RFI			
Treatment	Conc.	Control	MFI	Viability (%)	MFI	Corrected MFI	vs. Media	vs. DMSO	Viability (%)	MFI	Corrected MFI	vs. Media	vs. DMSO	Viability (%)	
Media	NA	Media	2.27	97.3	2.63	0.36	100	*NA*	96.6	2.66	0.39	100	*NA*	97.7	97.2
DMSO	0.2%	DMSO	2.64	97.5	3.15	0.51	142	100	97.0	2.90	0.26	67	100	97.7	97.4
DNCB	4 µg/ml		2.59	96.2	4.50	1.91	*NA*	375 ✶	96.1	3.61	1.02	*NA*	392 ✶	95.8	96.0
Nickel Sulfate	100 µg/ml	Media	2.98	94.7	5.01	2.03	564 ✶	*NA*	93.7	4.53	1.55	397 ✶	*NA*	93.1	93.8
Lactic Acid	1000 µg/ml		2.15	96.3	2.44	0.29	81	*NA*	96.6	2.36	0.21	54	*NA*	97.4	96.8
JA900-DAA (Lot PT-917-59)	1395.8 µg/ml	Media	2.79	97.5	3.88	1.09	303 ✶	*NA*	98.1	3.65	0.86	221 ✶	*NA*	97.8	97.8
	1666.7 µg/ml		2.96	97.3	3.35	0.39	108	*NA*	97.7	3.39	0.43	110	*NA*	97.2	97.4
	2000 µg/ml		2.72	97.1	3.92	1.20	333 ✶	*NA*	97.6	3.34	0.62	159	*NA*	97.5	97.4
	2395.9 µg/ml		2.97	97.8	3.51	0.54	150 ✶	*NA*	97.3	3.18	0.21	54	*NA*	97.4	97.5
	2875 µg/ml		2.95	97.0	3.45	0.50	139	*NA*	97.8	3.33	0.38	97	*NA*	97.7	97.5
	3458.4 µg/ml		2.68	96.3	3.63	0.95	264 ✶	*NA*	96.6	2.98	0.30	77	*NA*	96.7	96.5
	4166.7 µg/ml		2.77	96.7	3.59	0.82	228 ✶	*NA*	97.0	3.09	0.32	82	*NA*	96.8	96.8
	5000 µg/ml		2.78	96.9	3.52	0.74	206 ✶	*NA*	96.9	3.29	0.51	131	*NA*	96.5	96.8

✶ = positive sensitizing response (RFI of 150 or more for CD86, or RFI of 200 or more for CD54)
NA = not applicable
1 = mean of Isotype viability, CD86 viability and CD54 viability

All acceptance criteria were met

Table 3: Main Tests - Experimental Data (continued)

Main Test 2			Isotype		CD86					CD54					Mean[1] Viability (%)
							RFI					RFI			
Treatment	Conc.	Control	MFI	Viability (%)	MFI	Corrected MFI	vs. Media	vs. DMSO	Viability (%)	MFI	Corrected MFI	vs. Media	vs. DMSO	Viability (%)	
Media	NA	Media	1.97	96.6	2.84	0.87	100	*NA*	94.9	2.54	0.57	100	*NA*	96.4	96.0
DMSO	0.2%	DMSO	2.11	95.7	3.48	1.37	157 ✶	100	96.2	2.86	0.75	132	100	95.8	95.9
DNCB	4 µg/ml		2.06	41.3	3.77	1.71	*NA*	125 [2]	43.6	2.86	0.80	*NA*	107 [2]	40.7	41.9[3]
Nickel Sulfate	100 µg/ml	Media	2.15	93.9	4.16	2.01	231 ✶	*NA*	93.9	3.84	1.69	296 ✶	*NA*	93.3	93.7
Lactic Acid	1000 µg/ml		1.61	96.4	2.36	0.75	86	*NA*	93.7	2.17	0.56	98	*NA*	96.6	95.6
JA900-DAA (Lot PT-917-59)	1395.8 µg/ml	Media	2.16	95.5	2.94	0.78	90	*NA*	96.0	2.52	0.36	63	*NA*	94.9	95.5
	1666.7 µg/ml		1.93	95.8	2.65	0.72	83	*NA*	95.9	2.34	0.41	72	*NA*	95.9	95.9
	2000 µg/ml		1.95	96.8	2.57	0.62	71	*NA*	95.3	2.46	0.51	89	*NA*	96.3	96.1
	2395.9 µg/ml		1.77	96.9	2.47	0.70	80	*NA*	96.4	2.24	0.47	82	*NA*	96.8	96.7
	2875 µg/ml		1.75	96.7	2.83	1.08	124	*NA*	96.9	2.24	0.49	86	*NA*	96.3	96.6
	3458.4 µg/ml		1.86	95.9	2.60	0.74	85	*NA*	96.2	2.48	0.62	109	*NA*	96.2	96.1
	4166.7 µg/ml		1.86	96.0	2.65	0.79	91	*NA*	96.0	2.63	0.77	135	*NA*	95.8	95.9
	5000 µg/ml		1.80	95.6	2.66	0.86	99	*NA*	95.8	2.57	0.77	135	*NA*	95.5	95.6

✶ = positive sensitizing response (RFI of 150 or more for CD86, or RFI of 200 or more for CD54)
NA = not applicable
1 = mean of Isotype viability, CD86 viability and CD54 viability
2 = no positive response, failed to meet the acceptance criterion
3 = failed to meet the acceptance criterion of at least 50%

The DMSO vehicle control (for DNCB) had an RFI versus media of greater than 150, which failed to meet the acceptance criteria.
The DNCB positive control mean cell viability was less than 50%, which failed the acceptance criteria of greater than 50%. Also, the CD86 RFI was less than 150, and the CD54 RFI was less than 200, which failed to meet the acceptance criteria.

Table 3: Main Tests - Experimental Data (continued)

Main Test 3			Isotype		CD86					CD54					Mean[1] Viability (%)
							RFI					RFI			
Treatment	Conc.	Control	MFI	Viability (%)	MFI	Corrected MFI	vs. Media	vs. DMSO	Viability (%)	MFI	Corrected MFI	vs. Media	vs. DMSO	Viability (%)	
Media	NA	Media	2.11	96.4	3.12	1.01	100	*NA*	95.4	2.31	0.20	100	*NA*	96.4	96.1
DMSO	0.2%	DMSO	2.02	96.3	3.33	1.31	130	100	96.3	2.40	0.38	190	100	96.8	96.5
DNCB	4 µg/ml		2.60	49.9	5.25	2.65	*NA*	202✶	53.8	6.67	4.07	*NA*	1071✶	50.2	51.3
Nickel Sulfate	100 µg/ml	Media	2.03	93.8	4.29	2.26	224✶	*NA*	93.8	4.41	2.38	1190 ✶	*NA*	94.6	94.1
Lactic Acid	1000 µg/ml		1.75	93.7	2.40	0.65	64	*NA*	94.8	1.96	0.21	105	*NA*	95.8	94.8
JA900-DAA (Lot PT-917-59)	1395.8 µg/ml	Media	1.84	96.4	2.63	0.79	78	*NA*	97.2	2.12	0.28	140	*NA*	96.2	96.6
	1666.7 µg/ml		1.82	96.6	2.70	0.88	87	*NA*	96.7	2.03	0.21	105	*NA*	97.1	96.8
	2000 µg/ml		1.86	94.6	2.93	1.07	106	*NA*	95.2	2.05	0.19	95	*NA*	96.5	95.4
	2395.9 µg/ml		1.92	96.3	2.58	0.66	65	*NA*	96.3	2.01	0.09	45	*NA*	96.5	96.4
	2875 µg/ml		1.82	96.5	2.57	0.75	74	*NA*	96.7	2.00	0.18	90	*NA*	96.9	96.7
	3458.4 µg/ml		1.85	95.8	2.69	0.84	83	*NA*	96.7	2.12	0.27	135	*NA*	96.7	96.4
	4166.7 µg/ml		1.78	95.6	2.69	0.91	90	*NA*	95.9	2.14	0.36	180	*NA*	96.2	95.9
	5000 µg/ml		1.85	94.5	2.87	1.02	101	*NA*	95.2	2.16	0.31	155	*NA*	96.1	95.3

✶ = positive sensitizing response (RFI of 150 or more for CD86, or RFI of 200 or more for CD54)
NA = not applicable
1 = mean of Isotype viability, CD86 viability and CD54 viability

All acceptance criteria were met

Table 3: Main Tests - Experimental Data (continued)

Main Test 4			Isotype		CD86					CD54					Mean[1] Viability (%)
							RFI					RFI			
Treatment	Conc.	Control	MFI	Viability (%)	MFI	Corrected MFI	vs. Media	vs. DMSO	Viability (%)	MFI	Corrected MFI	vs. Media	vs. DMSO	Viability (%)	
Media	NA	Media	2.17	97.3	3.67	1.50	100	*NA*	93.7	2.71	0.54	100	*NA*	95.5	95.5
DMSO	0.2%	DMSO	2.10	96.8	3.67	1.57	105	100	96.9	2.58	0.48	89	100	96.9	96.9
DNCB	4 µg/ml		1.91	54.7	7.49	5.58	*NA*	355 ✶	56.6	6.22	4.31	*NA*	898 ✶	47.6	53.0
Nickel Sulfate	100 µg/ml	Media	2.31	86.6	5.39	3.08	205 ✶	*NA*	85.7	5.00	2.69	498 ✶	*NA*	88.3	86.9
Lactic Acid	1000 µg/ml		1.86	97.2	2.65	0.79	53	*NA*	96.4	2.21	0.35	65	*NA*	96.8	96.8
JA900-DAA (Lot PT-917-59)	1391.5 µg/ml	Media	2.52	93.5	3.37	0.85	57	*NA*	96.5	2.83	0.31	57	*NA*	95.7	95.2
	1670.5 µg/ml		2.32	93.6	3.24	0.92	61	*NA*	96.4	2.81	0.49	91	*NA*	95.4	95.1
	2005.4 µg/ml		2.43	92.4	3.17	0.74	49	*NA*	96.4	2.93	0.50	93	*NA*	95.6	94.8
	2407.4 µg/ml		2.32	94.4	3.58	1.26	84	*NA*	95.9	2.94	0.62	115	*NA*	95.5	95.3
	2890.1 µg/ml		2.38	93.5	3.48	1.10	73	*NA*	94.1	3.04	0.66	122	*NA*	94.4	94.0
	3469.5 µg/ml		1.95	96.1	3.07	1.12	75	*NA*	95.4	2.70	0.75	139	*NA*	96.0	95.8
	4165 µg/ml		1.99	95.6	3.46	1.47	98	*NA*	95.9	2.80	0.81	150	*NA*	95.6	95.7
	5000 µg/ml		2.10	96.2	3.38	1.28	85	*NA*	93.1	2.74	0.64	119	*NA*	95.8	95.0

✶ = positive sensitizing response (RFI of 150 or more for CD86, or RFI of 200 or more for CD54)
NA = not applicable
1 = mean of Isotype viability, CD86 viability and CD54 viability

All acceptance criteria were met

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SPONSOR
TEST ARTICLE CHARACTERIZATION INFORMATION

In compliance with Good Laboratory Practice (GLP) regulations, a characterization of the test article is required and should include identity, strength, purity, composition, stability and uniformity. This data must be reviewed by the Study Director prior to study termination and will be included in the final report. (EPA 40 CFR 160.105 and 792.105; FDA 21 CFR 58.105, OECD 6.2).

In addition, the test article characterization should be performed in compliance with the Good Laboratory Practices.

Any exceptions to the GLP requirements will be indicated in the Compliance Statement of the final report.

Accordingly, please supply the following information for each test article submitted:

Proprietary is defined for this form as known by the Sponsor, but confidential.

Please do not use NA for any portion of this form.

Test Article Identity JA900-DAA

Lot/Batch# PT-917-59

Stability (Duration) expire March 2017 ☐ Unknown ■ Proprietary

Storage ■ Room Temperature ☐ Refrigerated (2-8°) ☐ Other: protected from light

Strength Polymer in ethanol at 51% ☐ Unknown ■ Proprietary

Purity 51% ☐ Unknown ■ Proprietary

Composition 51% Polymer (Mn =1047); 49% ethanol ☐ Unknown ■ Proprietary

Uniformity uniform ☐ Unknown ■ Proprietary

☐ This characterization **was** conducted under GLPs

☐ This characterization **was** conducted under GMPs

■ This characterization **was not** conducted under GLPs **or** GMPs.

BY: Xiao Huang/ June 22, 2016 (signature) (date) FOR: IFF (company)

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Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	1-CHLORO-2,4-DINITROBENZENE 97 %
Product Number:	138630
Batch Number:	STBF4847V
Brand:	Aldrich
CAS Number:	97-00-7
Formula:	$ClC_6H_3(NO_2)_2$
Formula Weight:	202.55
Quality Release Date:	31 MAR 2015

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	LIGHT YELLOW TO BROWN	LIGHT YELLOW
APPEARANCE (FORM)	CRYSTALS OR CRYSTALLINE CHUNK(S) OR CHUNK(S) OR SOLID	CRYSTALS WITH CHUNK(S)
PURITY (GC AREA %)	≥ 97.5 %	> 99.9 %
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS
PROTON NMR SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

Dr. Claudia Geitner
Manager Quality Control
Steinheim, Germany

Version	0
Molecular weight	262.85
Molecular formula	Ni O4 S . 6 H2 O
CAS No	10101-97-0
Linear formula	NiSO4.6H2O
Flash point (°C)	

Certificate of Analysis

This is to certify that units of the below mentioned lot number were tested and found to comply with the specifications of the grade listed. Certain data have been supplied by third parties. Acros Organics expressly disclaims all warranties, expressed or implied, including the implied warranties of merchantability and fitness for a particular purpose. Unless otherwise stated, these products are not intended for dialysis, parenteral or injectable use without further processing. The following are the actual analytical results obtained:

Catalog Number	21108	**Quality Test / Release Date**	26 March 2015
Lot Number	A0357363	**Suggested Retest Date**	March 2020
Description	Nickel(II) sulfate hexahydrate,99%,for analysis		
Country of Origin	BELGIUM		
Declaration of Origin	synthetic		

Origin Comment	

Result Name	Specifications	Test Value
Appearance	blue-green crystals	blue-green crystals
Titration Complexometric	>=98.5 %	98.9 %
pH	4 to 6 (5% soln. at 20°C)	4.4 (5% soln. at 20°C)
Chloride (Cl)	=<50 ppm	=<5 ppm
Arsenic (As)	=<10 ppm	1 ppm
Cadmium (Cd)	=<50 ppm	1 ppm
Cobalt (Co)	=<100 ppm	5 ppm
Copper (Cu)	=<20 ppm	1 ppm
Iron (Fe)	=<50 ppm	5 ppm
Lead (Pb)	=<20 ppm	1 ppm
Zinc (Zn)	=<50 ppm	1 ppm

L. Van den Broek, QA Manager Issued: 20 October 2016

Acros Organics
ENA23, zone 1, nr 1350, Janssen Pharmaceuticalaan 3a, B-2440 Geel, Belgium
Tel +32 14/57.52.11 - Fax +32 14/59.34.34 Internet: http://www.acros.com
1 Reagent Lane, Fair Lawn, NJ 07410,USA Fax 201-796-1329

SIGMA-ALDRICH

3050 Spruce Street, Saint Louis, MO 63103 USA
Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	LACTIC ACID Ph Eur
Product Number:	69775
Batch Number:	BCBM8154V
Brand:	Fluka
CAS Number:	50-21-5
Formula:	$CH_3CH(OH)COOH$
Formula Weight:	90.08
Quality Release Date:	07 MAY 2014
Recommended Retest Date:	FEB 2018

TEST	SPECIFICATION	RESULT
PHARMACOPOEA TESTS	CORRESPONDS TO REQUIREMENTS	CORRESPONDS TO PH.EUR.8.1
IDENTIFICATION A	SOLUTION IS STRONGLY ACIDIC	CORRESPONDS
IDENTIFICATION B	RELATIVE DENSITY 1.20 - 1.21	1.21
IDENTIFICATION C	REACTION OF LACTATES	CORRESPONDS
APPEARANCE OF SOLUTION	NOT MORE INTENSELY COLORED THAN REFERENCE SOLUTION Y6	CORRESPONDS
ETHER-INSOLUBLE SUBSTANCES	NOT MORE OPALESCENT THAN THE SOLVENT USED FOR THE TEST	CORRESPONDS
SUGARS AND OTHER REDUCING SUBSTANCES	NO RED OR GREENISH PRECIPITATE IS FORMED	CORRESPONDS
CITRIC, OXALIC AND PHOSPHORIC ACIDS	CORRESPONDS	CORRESPONDS
RESIDUAL SOLVENTS	CORRESPONDS	CORRESPONDS
CALCIUM	MAX. 200 PPM	<1 PPM
HEAVY METALS	MAX. 10 PPM	<5 PPM
SULPHATES	MAX. 200 PPM	<5 PPM
SULPHATED ASH	MAX. 0.1 %	<0.001 %
ASSAY	88.0 - 92.0 % (M/M)	89.6 %
REMARKS	–	NOT TESTED FOR USE IN THE MANUFACTURE OF PARENTERAL DOSAGE FORMS

Dr. Claudia Geitner
Manager Quality Control
Buchs, Switzerland

Fisher Scientific

1 Reagent Lane
Fair Lawn, NJ 07410
201.796.7100 tel
201.796.1329 fax

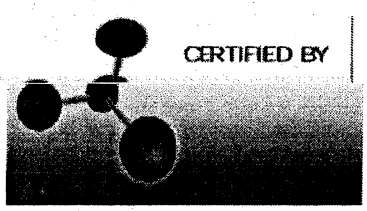
Certificate of Analysis

Fisher Scientific's Quality System has been found to conform to Quality Management System Standard ISO9001:2008 standard by SAI Global Certificate Number CERT - 0090918

This is to certify that units of the lot number below were tested and found to comply with the specifications of the grade listed. Certain data have been supplied by third parties. Fisher Scientific expressly disclaims all warranties, expressed or implied, including the implied warranties of merchantability and fitness for a particular purpose. Certain products (USP/FCC/NF/EP/BP/JP grades) are sold for use in food, drug, or medical device manufacturing. Fisher does not claim regulatory coverage under 21 CFR nor maintain DMF's with the FDA. The following are the actual analytical results obtained:

Catalog Number	**D128**	**Quality Test / Release Date**	**4/1/2016**
Lot Number	**161028**		
Description	**DIMETHYLSULFOXIDE, A.C.S.**		
Country of Origin	**United States**	*** Suggested Retest Date**	**Mar-2021**
Chemical Origin	**Organic - non animal**		
BSE/TSE Comment	No animal products are used as starting raw material ingredients, or used in processing, including lubricants, processing aids, or any other material that might migrate to the finished product.		

Result name	Units	Specifications	Test Value
APPEARANCE		REPORT	CLEAR, COLORLESS LIQUID
ASSAY	%	>= 99.9	99.9
DENSITY AT 25 DEGREES C	GM/ML	>= 1.095	1.095
EVAPORATION RESIDUE	%	<= 0.01	<0.001
IDENTIFICATION	PASS/FAIL	= PASS TEST	PASS TEST
TITRATABLE ACID	mEq/g	<= 0.001	<0.0002
WATER (H2O)	%	<= 0.1	0.04



Quality Control Manager BPF

Note: The data listed is valid for all package sizes of this lot of this product, expressed as a extension of this catalog number listed above. If there are any questions with this certificate, please call Chemical Services at (800) 227-6701.
*Based on suggested storage condition.

HOSPIRA, INC. - ROCKY MOUNT, NC

DATED: 13-08-26 W7138BQAC PAGE 1

REVW *13-04-05* EFFC *13-08-26* SUBTYPE-8 AREA-B
Desc: Certificate of Analysis Hospira, Inc. (Rocky Mount N.C.) BQ
Written By/Date: L. Debusk 4-11-13 Unit: SRB/Rocky Mount
Approved By/Date: BQ J. Miles 8-19-13

Specification No.: 60.07138ALLCODE, QPO.18.004

Technical Note:

1. This product has been manufactured and tested in current good manufacturing practices (CGMP) facilities in accordance with appropriate regulations. This product meets applicable specifications, applicable regulatory submissions or marketing authorizations and, where appropriate, compendial requirements. The undersigned certifies this to be a true representation of the results.

==========

Product Test Results

STEP/PROCEDURE	DOC. REF.	RESULTS	SIGNATURE/DATE
I. Biological Requirements:			
A. Bacterial Endotoxin Test: Not more than 0.50 EU/ML.	90.B-0610	<0.06 EU/ML	03/28/14
B. Solution Bioburden Must meet requirements	QPO.29.001 WGENVIRO	Pass ✓ Fail ___	03/28/14
			03/28/14 BQ REVIEW

****

END OF DOCUMENT

7138-04-39 1000 ML 3 38-603-4B EXP. DATE 1FEB2017 JD17032
0.9% SODIUM CHLORIDE IRRIGATION, USP

COMMENTS:

ISSUER: MCQUEJT 01/30/14

HOSPIRA, INC. - ROCKY MOUNT, NC
DATED: 09-09-25 W7138CQA PAGE 1

REVW *09-06-05* EFFC *09-09-25* SUBTYPE-7 AREA-T
Desc: Certificate of Analysis Hospira, Inc. (Rocky Mount N.C.) CQ
Written By/Date: B. Catlett 8-30-09 Unit: SRB/Rocky Mount
Specification Comparison Completed by/date: CQ: D. Short 9-1-09
BRQ: R. Slade 8-10-09

Specification No.: QPO.18.004

Technical Note:
1. This product has been manufactured and tested in current good manufacturing practices (CGMP) facilities in accordance with appropriate regulations. This product meets applicable specifications, applicable regulatory submissions or marketing authorizations and, where appropriate, compendial requirements. The undersigned certifies this to be a true representation of the results.

Product Manufacturing Information

Date of Manufacture: 2/5/14 Batch Size 360000L (NDC) DIN No.: 0409-7138-09 ~~0409-7138-09~~ write over

STEP/PROCEDURE	DOC. REF.	SIGNATURE/DATE
Manufacturing Formula	92.D-7138 Current Date: 3/25/08	
Commodity and Process Summary	35. 07138043G Current Date: 2/23/10	
Printed Material Summary	40. 07138044G Current Date: 11/3/09	
Sampling and Testing Requirements	60.07138ALLCODE Current Date: 10/12/10	3/28/14

7138-04-39 1000 ML 3 38-603-4B EXP. DATE 1FEB2017 JD17032
0.9% SODIUM CHLORIDE IRRIGATION, USP

COMMENTS:

ISSUER: MCQUEJT 01/30/14

HOSPIRA, INC. - ROCKY MOUNT, NC
DATED: 09-09-25 W7138CQA PAGE 2

PRODUCT TEST RESULTS

STEP/PROCEDURE	DOC. REF.	RESULTS	SIGNATURE/DATE
I. Batch Release System			
A. Clarity	90.P-0363		
1. Solution must be clear		Pass ✓ Fail	
2. Solution must not contain one or more particles which are visible upon attentive examination			
B. Volume Between 1000 to 1060 ml Between 1500 to 1560 ml	90.P-0081	1035 ml Ave	
C. Sterility Must meet requirements of parametric release.	90.M-0477	Pass ✓ Fail	JSpurlock 3/28/14 BRQ REVIEW
II. Chemical Requirements			
A. Sodium Chloride Final product limits: 95.0% to 105.0%	90.C-0042	99.5%	
B. Heavy Metals Not more than 10 PPM:	90.C-1221	Not more than	10 PPM
C. Iron Final product limits: Not more than 2 PPM	90.C-0869 (0.0002%)	Not more than	2PPM (0.0002%)
D. Identification: Responds to test for:	90.C-1648		
Sodium	90.C-1648	Pass ✓ Fail	
Chloride	90.C-0003	Pass ✓ Fail	

7138-04-39 1000 ML 3 38-603-4B EXP. DATE 1FEB2017 JD17032
0.9% SODIUM CHLORIDE IRRIGATION, USP

COMMENTS:

ISSUER: MCQUEJT 01/30/14

HOSPIRA, INC. - ROCKY MOUNT, NC
DATED: 09-09-25 W7138CQA PAGE 3

STEP/PROCEDURE	DOC. REF.	RESULTS	SIGNATURE/DATE
E. pH Final product limits: Between 4.5 and 7.0	90.C-0021	5.7 pH @ 24.9°C	A. Oldens 02/17/14 CQ REVIEW
F. Residual Solvents Meets USP <467>	N/A	No Class 1, Class 2, Class 3 or other solvents used. Drug product testing is not required.	

ER-HIGH yes ( ) No (✓) Check applicable box ER No. ——— S Spruill 3/28/14

****

END OF DOCUMENT

7138-04-39 1000 ML 3 38-603-4B EXP. DATE 1FEB2017 JD17032
0.9% SODIUM CHLORIDE IRRIGATION, USP

COMMENTS:

ISSUER: MCQUEJT 01/30/14

HyClone™

CERTIFICATE OF ANALYSIS

Product: RPMI-1640 MEDIUM (1X)
+ 2.05 mM L-Glutamine

Lot #: AAK205984 **Catalog #:** SH30027 **Expiration Date:** OCT/2016

Test	Specification	Units	Results
Appearance	Clear reddish solution		Clear reddish solution
pH	7.0 - 7.4		7.0
Osmolality	260 - 290	mOsm/kg	278
Sterility Testing			
Bacteria and Fungi	No Growth		No Growth
Endotoxin	≤ 1.0	EU/mL	<0.01
Growth Promotion			Satisfactory

Cell growth was assessed over a minimum of three subculture generations. Cell cultures are observed for evidence of nutritional deficiency, cytotoxicity, or morphological aberrations. The product is tested in parallel with a control lot.

Cell Lines used: CHO-K1 Cells

Jessica Stauffer
Quality Control Department Signature

14NOV2015
Date

GE Healthcare Lifesciences
HyClone Laboratories, Inc.
925 West 1800 South Logan, Utah 84321 USA T 435 792 8000 F 435 792 8011

HyClone™

CERTIFICATE OF ANALYSIS

Product: RPMI-1640 MEDIUM (1X)
+ 2.05 mM L-Glutamine

Lot #: ABB213926 **Catalog #:** SH30027 **Expiration Date:** FEB/2017

Test	Specification	Units	Results
Appearance	Clear reddish solution		Clear reddish solution
pH	7.0 - 7.4		7.1
Osmolality	260 - 290	mOsm/kg	273
Sterility Testing			
Bacteria and Fungi	No Growth		No Growth
Endotoxin	≤ 1.0	EU/mL	<0.01
Growth Promotion			Satisfactory

Cell growth was assessed over a minimum of three subculture generations. Cell cultures are observed for evidence of nutritional deficiency, cytotoxicity, or morphological aberrations. The product is tested in parallel with a control lot.

Cell Lines used: CHO-K1 Cells

Bradden Smith
Quality Control Department Signature

03 MAR 2016
Date

GE Healthcare Lifesciences
HyClone Laboratories, Inc.
925 West 1800 South Logan, Utah 84321 USA T 435 792 8000 F 435 792 8011

SIGMA-ALDRICH®

3050 Spruce Street, Saint Louis, MO 63103 USA
Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	HEPES BUFFER SOLUTION 1M in water
Product Number:	83264
Batch Number:	BCBR0190V
Brand:	Sigma
CAS Number:	
Formula:	$C_8H_{18}N_2O_4S$
Formula Weight:	238.30
Quality Release Date:	28 JAN 2016
Date retested:	12 JUL 2016
Recommended Retest Date:	APR 2017

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	COLORLESS	COLORLESS
APPEARANCE (FORM)	LIQUID	LIQUID
DENSITY D20/4	1.071 - 1.075	1.073
REFRACTIVE INDEX N20/D	1.370 - 1.372	1.371
PH	5.5 +/- 0.5	5.2
PROTON NMR SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS
EXTRANEOUS ACTIVITIES	DNASES, RNASES, PROTEASES, PHOSPHATASES NOT DETECTABLE	DNASES, RNASES, PROTEASES, PHOSPHATASES NOT DETECTABLE
RESIDUE (FILTER TEST)	NO RESIDUE	NO RESIDUE

Dr. Claudia Geitner
Manager Quality Control
Buchs, Switzerland

Fisher Scientific

1 Reagent Lane
Fair Lawn, NJ 07410
201.796.7100 tel
201.796.1329 fax

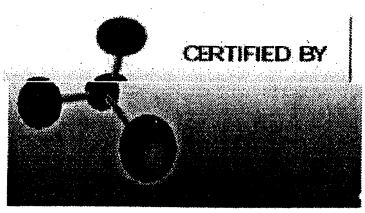
Certificate of Analysis

Fisher Scientific's Quality System has been found to conform to Quality Management System Standard ISO9001:2008 standard by SAI Global Certificate Number CERT - 0090918

This is to certify that units of the lot number below were tested and found to comply with the specifications of the grade listed. Certain data have been supplied by third parties. Fisher Scientific expressly disclaims all warranties, expressed or implied, including the implied warranties of merchantability and fitness for a particular purpose. Certain products (USP/FCC/NF/EP/BP/JP grades) are sold for use in food, drug, or medical device manufacturing. Fisher does not claim regulatory coverage under 21 CFR nor maintain DMF's with the FDA. The following are the actual analytical results obtained:

Catalog Number	**BP2959**	**Quality Test / Release Date**	**3/30/2016**
Lot Number	**159063**	**Expiration Date**	**Aug/17**
Description	**PENICILLIN/STREPTOMYCIN MIXTURE**		
Country of Origin	**United States**		

Result name	Units	Specifications	Test Value
APPEARANCE		REPORT	Clear solution
PENICILLIN	U/ML	= 10000	10000
PH		Inclusive Between 5.0 - 7.0	6.5
SODIUM CHLORIDE	%	= 0.90	0.90
STERILE FILTERED	PASS/FAIL	= PASS TEST	PASS TEST
STREPTOMYCIN	mg/ml	= 10	10



Quality Control Manager BPF

Note: The data listed is valid for all package sizes of this lot of this product, expressed as a extension of this catalog number listed above. If there are any questions with this certificate, please call Chemical Services at (800) 227-6701.
*Based on suggested storage condition.

ThermoFisher SCIENTIFIC

The world leader in serving science

CERTIFICATE OF ANALYSIS

PRODUCT NAME: 2-Mercaptoethanol

PRODUCT NUMBER: 35602BID

LOT NUMBER: QC215754A

SPECIFICATIONS:

VISUAL: Clear, colorless to very faint yellow liquid, free of particulate matter.

PURITY: ≥ 99%

RESULTS:

VISUAL: Clear, colorless to very faint yellow liquid, free of particulate matter.

PURITY: 99.9 %

Publish Date: 3/24/2015

Liam F. Garrity, Senior Manager, Analytical Services Laboratory

[Rev.001]

Pierce Biotechnology | PO Box 117 | (800) 874-3723 tel | (815) 968-7316 fax
3747 N. Meridian Road | Rockford, IL 61105 USA | (815) 968-0747 tel | www.thermoscientific.com/pierce

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Water, sterile-filtered, BioReagent, suitable for cell culture
Product Number	W3500
Product Brand	SIGMA
CAS Number	7732-18-5
Molecular Formula	H_2O
Molecular Weight	18.02

TEST	SPECIFICATION	LOT rnbf0858 RESULTS
Storage:		ROOM TEMPERATURE
Print Date:		23 NOV 2015
Expiry date:		NOV 2017
Date of QC Release:		23 NOV 2015
Place of Manufacture:		Irvine. United Kingdom
Production Date:		NOV 2015
Appearance (Turbidity)	Clear	Clear
Appearance (Form)	Liquid	Liquid
pH	5.0 - 7.0	6.8
Sterility	Pass	Pass
Endotoxin Level	<= 1 EU/ml	< 1 EU/ml
Cell Culture Testing - MTT	Pass	Pass
Cell Line	Cell Line - Cell Types	Vero

Jane Findlay

Jane Findlay, Manager
Quality Control
Irvine United Kingdom

SERUM SOURCE INTERNATIONAL

Serum Source International, Inc.
386 Crompton Street • Charlotte, NC 28273
USA

Toll free 888•588•8115
Phone 704•588•6607
Fax 704•588•6608

CERTIFICATE OF ANALYSIS

Product: FETAL BOVINE SERUM
USA ORIGIN
Triple 0.1µm Sterile Filtered
Heat Inactivated

Lot No: FBU15678HI
Expires: 09/2019
Catalog Numbers: FB02-50HI, FB02-100HI, FB02-500HI

This product is for further manufacturing use. It is not intended for human or animal therapeutic use. This serum was processed at FDA and/or USDA licensed facilities and collected from abattoirs inside the United States inspected and approved by the United States Department of Agriculture from bovine fetuses derived from healthy animals, which received ante-mortem and post-mortem inspections and were found to be free of signs and symptoms of infectious and contagious diseases.

ELECTROPHORESIS	Results
Electrophoretic Pattern	Normal
Total Protein	3.7 g/dL

PHYSICAL AND CHEMICAL ANALYSIS	Results
Endotoxin	< 0.1 EU/mL
Hemoglobin	12.5 mg/dL
Osmolality	304 mOsm/Kg
pH	7.65
Protein	3.7 g/dL

Albumin	1.9 g/dL
Alpha 1,2	1.4 g/dL
ALP	161 IU/L
ALT (SGPT)	7 IU/L
AST (SGOT)	25 IU/L
Bilirubin	0.2 mg/dL
BUN	14 mg/dL
Calcium	13.8 mg/dL
Chloride	98 mEq/L
Cholesterol	36 mg/dL
Creatinine	2.4 mg/dL
GGT	4 IU/L
Globulin (Beta)	0.3 g/dL
Globulin (Gamma)	0.0 g/dL
Glucose	93 mg/dL
IgG	79.3 µg/mL
Iron	214 µg/dL
Magnesium	3.1 mg/dL
Phosphorus	9.9 mg/dL
Potassium	> 10.0 mEq/L
Sodium	134 mEq/L
Triglyceride	78 mg/dL
Uric Acid	2.2 mg/dL

SERUM SOURCE INTERNATIONAL

Serum Source International, Inc.
386 Crompton Street • Charlotte, NC 28273
USA

Toll free 888•588•8115
Phone 704•588•6607
Fax 704•588•6608

Product: FETAL BOVINE SERUM, USA ORIGIN, Triple 0.1µm Sterile Filtered, Heat Inactivated
Lot No.: FB0115678HI
Expires: 09-2019
Catalog Numbers: FB02-50HI, FB02-100HI, FB02-500HI

VIRUS AND ANTIBODY TESTING – (9 CFR 113.53)		Specifications	Results
BAV	(Bovine Adenovirus)	not detected	not detected
BRSV	(Bovine Respiratory Syncytial Virus)	not detected	not detected
BVD	(Bovine Virus Diarrhea)	not detected	not detected
BPV	(Bovine Papillomavirus)	not detected	not detected
BTV	(Bluetongue Virus)	not detected	not detected
IBR	(Infectious Bovine Rhinotracheitis)	not detected	not detected
PI3	(Parainfluenza-3)	not detected	not detected
Rabies		not detected	not detected
REO	(Reovirus)	not detected	not detected

MICRO-ORGANISM TESTING	Specifications	Results
Bacteria and Fungi	not detected	not detected
Mycoplasma	not detected	not detected
Sterility	no evidence of microbial growth	no evidence of microbial growth

GROWTH PERFORMANCE TESTING CELL LINE: L929, VERO	Specifications	Results
Population	> 75 %	pass
Relative Growth Promotion	> 75 %	pass

HORMONE TESTING	Results	
Testosterone	<0.01	ng/mL
Estradiol	0.00	pg/mL
Insulin	6.75	µIU/mL
Cortisol	0.00	µg/dL
Progesterone	<0.1	ng/mL
T3	88.8	ng/dL
T4	9.4	µg/dL

SERUM SOURCE INTERNATIONAL

Serum Source International, Inc.
386 Crompton Street • Charlotte, NC 28273
USA

Toll free 888•588•8115
Phone 704•588•6607
Fax 704•588•6608

CERTIFICATE OF ANALYSIS

Product:	**FETAL BOVINE SERUM**	**Lot No:**	**FBU15680HI**
	USA ORIGIN	**Expires:**	**11-2020**
	Triple 0.1µm Sterile Filtered	**Catalog Numbers:**	**FB02-50HI, FB02-100HI, FB02-500HI**
	Heat Inactivated		

This product is for further manufacturing use. It is not intended for human or animal therapeutic use. This serum was processed at FDA and/or USDA licensed facilities and collected from abattoirs inside the United States inspected and approved by the United States Department of Agriculture from bovine fetuses derived from healthy animals, which received ante-mortem and post-mortem inspections and were found to be free of signs and symptoms of infectious and contagious diseases.

ELECTROPHORESIS	Results
Electrophoretic Pattern	Normal
Total Protein	3.6 g/dL

PHYSICAL AND CHEMICAL ANALYSIS	Results
Endotoxin	<0.1 EU/mL
Hemoglobin	9.12 mg/dL
Osmolality	297 mOsm/Kg
pH	7.29
Protein	3.6 g/dL

Albumin	2.1 g/dL
Alpha 1,2	1.2 g/dL
ALP	184 IU/L
ALT (SGPT)	4 IU/L
AST (SGOT)	42 IU/L
Bilirubin	0.2 mg/dL
BUN	13 mg/dL
Calcium	13.4 mg/dL
Chloride	101 mEq/L
Cholesterol	31 mg/dL
Creatinine	2.9 mg/dL
GGT	4 IU/L
Globulin (Beta)	0.3 g/dL
Globulin (Gamma)	0.0 g/dL
Glucose	110 mg/dL
IgG	117.9 µg/mL
Iron	176 µg/dL
Magnesium	2.9 mg/dL
Phosphorus	10.0 mg/dL
Potassium	> 10.0 mEq/L
Sodium	138 mEq/L
Triglyceride	55 mg/dL
Uric Acid	2.3 mg/dL

SERUM SOURCE INTERNATIONAL

Serum Source International, Inc.
386 Crompton Street • Charlotte, NC 28273
USA

Toll free 888•588•8115
Phone 704•588•6607
Fax 704•588•6608

Product: FETAL BOVINE SERUM
USA ORIGIN
Triple 0.1µm Sterile Filtered
Heat Inactivated

Lot No: FBU15680HI
Expires: 11-2020
Catalog Numbers: FB02-50HI, FB02-100HI, FB02-500HI

VIRUS AND ANTIBODY TESTING – (9 CFR 113.53)		Specifications	Results
BAV	(Bovine Adenovirus)	not detected	not detected
BRSV	(Bovine Respiratory Syncytial Virus)	not detected	not detected
BVD	(Bovine Virus Diarrhea)	not detected	not detected
BPV	(Bovine Papillomavirus)	not detected	not detected
BTV	(Bluetongue Virus)	not detected	not detected
IBR	(Infectious Bovine Rhinotracheitis)	not detected	not detected
PI3	(Parainfluenza-3)	not detected	not detected
Rabies		not detected	not detected
REO	(Reovirus)	not detected	not detected

MICRO-ORGANISM TESTING	Specifications	Results
Bacteria and Fungi	not detected	not detected
Mycoplasma	not detected	not detected
Sterility	no evidence of microbial growth	no evidence of microbial growth

GROWTH PERFORMANCE TESTING CELL LINE: L929, VERO	Specifications	Results
Population	> 75 %	pass
Relative Growth Promotion	> 75 %	pass

HORMONE TESTING	Results	
Testosterone	<0.01	ng/mL
Estradiol	0.00	pg/mL
Insulin	4.29	µIU/mL
Cortisol	<0.2	µg/dL
Progesterone	<0.1	ng/mL
T3	118	ng/dL
T4	9.23	µg/dL

Human Cell Line Activation Test (h-CLAT)

Standard Protocol

MB Protocol Number: 705

Section 1. OBJECTIVE	
Objective:	To determine the skin sensitization potential of a test article as assessed by the measurement of surface markers (CD86, CD54) via flow cytometry. The h-CLAT is designed to detect sensitization induced by a test article in an *in vitro* sensitization assay using the THP-1 human monocytic cell line as the test system. The assay is based on research described in Takenouchi, et al. (2013), the EURL ECVAM DataBase service on Alternative Methods to animal experimentation (DB-ALM) Protocol No. 158, and the Draft OECD Guideline "*In Vitro* Skin Sensitisation: Human Cell Line Activation Test (h-CLAT)".
Basis of Method:	Cell viability will be obtained for each test article concentration by propidium iodide staining and flow cytometric analysis. For prediction of cytotoxicity and sensitization potential, the concentration responses obtained in the presence of test article will be compared, usually at the CV75 level, i.e., the concentration at which cell viability is approximately 75%. Any increases in CD86 and CD54 markers above vehicle control levels will be assessed to determine if the test article has sensitization potential around the CV75 dose levels. The test contains three parts: Reactivity check to ensure that the cells are growing adequately and performing properly Viability screen to determine the CV75 value Main test to determine CD86 and CD54 expression

Section 2. TEST ARTICLE	
Source:	All test articles will be supplied by the Sponsor. Prior to initiation of the study, the Sponsor should provide the Study Director with test article characterization.
Characterization:	Characterization of the test article is required in support of data submissions and should include identity, strength, purity, composition, stability and uniformity. These data must be reviewed by the Study Director prior to study initiation and included in the final report. (EPA 40 CFR 160.105 and 792.105; FDA 21 CFR 58.105, OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, No.1, Sect. 6.2.) When applicable, the lack of complete test article characterization will be addressed in the GLP compliance section of the report.
Label:	Each test article will be identified by source, name and/or code number, date of receipt at MB Research, and MB Project Number.
Safety Data Sheet:	If available, an SDS for each test article will be supplied by the Sponsor.
Storage:	Refer to Sponsor Request section.
Safety:	Based on the information provided by the Sponsor, appropriate routine safety precautions will be exercised in the handling of the test article.
Analysis:	Analysis of test articles in carriers (vehicles) for homogeneity and stability will not be performed unless requested by the Sponsor (at an additional cost). When applicable, the lack of analysis will be addressed in the GLP compliance section of the report.

Section 3. TEST SYSTEM	
Test System:	The immortalized human monocytic leukemia cell line, THP-1. Cells are available from the American Type Culture Collection (ATCC, cat. no.TIB-202) or from the European Collection of Cell Cultures (ECACC, cat. no. 88081201).
Justification:	THP-1 cells are used as a surrogate for human dendritic cells.
Thawing and Maintenance:	Cells will be maintained per MB Standard Operating Procedures.

Section 4. TEST ARTICLE PREPARATION	
Solubility:	Solubility will be checked at the following concentrations until a solution can be achieved: • 100 mg/ml in saline • 500 mg/ml in DMSO Or the highest soluble concentration (HSC) will be determined by diluting the 500 mg/ml by factors of two (250 mg/ml, 125 mg/ml, etc.) with a minimum concentration of 1 mg/ml.
Surfactants:	Saline will be used as the vehicle for testing surfactants. If not soluble at 100 mg/ml, the HSC will be determined as described above. (Sonication for approximately 5 minutes can be used to help solubilize the material, and other vehicles can be assessed if sufficient scientific rationale is provided.)
Preparation of Test Article Stock Solutions:	<u>Screen</u>: Eight concentrations of the test article will be tested. Concentrations will be prepared by diluting the stock test article solution in 1:1 serial dilutions starting from 100 mg/ml in saline, 500 mg/ml in DMSO, or the HSC.
	<u>Main Test</u>: The formulations will be freshly prepared prior to use. The highest concentration of the test article will be prepared first. All preparation procedures will be performed under yellow light. • If the test article is soluble in culture medium or saline, the solution will be prepared so that the final concentration is 100 times the concentration of 1.2 times the CV75 (1.2x CV75). (For example, if the CV75 is 50 µg/ml, 1.2x CV75 value is 60 µg/ml; therefore, 6 mg of the test article will be weighed and brought to a total volume of 1 ml with culture medium or saline.) • If the test article is soluble in DMSO, the solutions will be prepared so that the final concentration is 500 times the corresponding 1.2x CV75. • Note: If 1.2x CV75 is lower than 10 µg/ml, a 10 times (10x) higher concentrated stock solution will be prepared in order to be able to accurately weigh the test article. Once the 10x solution is made, it will be diluted (1:9 fold) for the preparation of the 1x highest dose stock solution. • Stock solutions for the additional seven concentrations will be prepared by $1/1.2^{x}$ serial dilutions (e.g., add 833 µl to a tube containing 167 µl of solvent, mix, repeat, etc.).

Section 4.	TEST ARTICLE PREPARATION (cont'd)
Preparation of Test Article Working Solutions:	All test article working solutions will be prepared by diluting stock solutions in culture medium. • For test articles soluble in saline, the stock solutions will be diluted (1:49 fold, e.g., 50 µl stock solution in 2450 µl of culture medium). • For test articles soluble in DMSO, the stock solutions will be diluted (1:249 fold, e.g., 10 µl of stock solution in 2490 µl of culture medium). Sonicate if precipitation is observed to ensure uniform distribution in culture medium (sonication should not exceed 5 minutes).
Disposition:	Any remaining test article solutions will be discarded after use. Unused test article will be discarded upon submission of the final report. A retention sample of the test article will not be retained.

Section 5.	CONTROLS, MEDIA, AND ASSAY SOLUTIONS
Materials:	• Sterile physiological saline, 0.9% (w/v) of NaCl (Sigma-Aldrich, cat. no. S8776, or equivalent) • Dimethylsulfoxide (DMSO) (Sigma-Aldrich, cat. no. 15140-122 or equivalent) • 2,4-Dinitrochlorobenzene (DNCB) (Sigma-Aldrich, cat. no. 138630, or equivalent) • Nickel Sulfate ($NiSO_4$) (Acros, cat. no. 211081000, or equivalent) • Lactic Acid (LA) (Fluka, cat. no. 69775, or equivalent) • RPMI-1640 medium (Gibco, cat. no. 22400-089, or equivalent) • 2-Mercaptoethanol (Thermo Scientific, cat. no. 35602, or equivalent) • HEPES buffer solution, 1 M (Sigma, cat. no. 83264, or equivalent) • Penicillin-Streptomycin (Fisher Scientific, cat. no. BP2959-50, or equivalent) • Fetal Bovine Serum (FBS), heat inactivated (Serum Source International, cat. no. FB02-500HI, or equivalent) • Bovine Albumin Fraction V (BSA), (Calbiochem, cat. no. 12660 or equivalent) • Dulbecco's Phosphate-buffered saline without magnesium, calcium, or phenol red (dPBS) (Gibco, cat. no. 14190-136, or equivalent) • Globulins Cohn fraction II, III, Human (Sigma, cat. no. G2388 or equivalent) • Propidium Iodide (PI), (Sigma-Aldrich, cat. no. P4170, or equivalent) • FITC-labeled mouse monoclonal anti-human CD86 antibody (Clone:Fun-1) (BD-PharMingen, cat. no. 555657, or equivalent) • FITC-labeled mouse monoclonal anti-human CD54 antibody (Clone: 6.5B5) (Dako, cat. no. F7143, or equivalent) • FITC-labeled mouse monoclonal IgG1 (Dako, cat. no. X0927, or equivalent)
Control Articles:	All control articles will be supplied by MB Research Labs. Control articles will be considered 100% active/pure for the purpose of dosage calculations. Lot/batch numbers, storage conditions and physical descriptions, will be documented in the raw data and included in the report. Control articles will be prepared fresh for each day of dosing, and any remaining control solutions will be discarded after use. All preparation procedures will be performed under yellow light.

Section 5.	CONTROLS, MEDIA, AND ASSAY SOLUTIONS (cont'd)
Solvent Controls:	Sterile physiological (0.9%) saline. If necessary, a test article can be prepared in DMSO at 500-fold the desired final concentration (generally 500 mg/ml). Saline Control: 100 µl of sterile physiological (0.9%) saline will be added to 4,900 µl of THP-1 culture medium. DMSO Control: 20 µl of DMSO will be added to 4,980 µl of THP-1 culture medium. This control will be used only if the test article is prepared in DMSO.
Positive Controls:	2,4-Dinitrochlorobenzene (DNCB, final test concentration 4 µg/ml) and nickel sulfate ($NiSO_4$, final test concentration 100 µg/ml). DNCB Stock Solution: A 2 mg/ml DNCB stock solution will be prepared. (For example, 10 mg (0.01 g) of DNCB will be weighed in a sterile tube or volumetric flask and brought to a total volume of 5 ml with DMSO.) DNCB Working Solution: An 8 µg/ml DNCB working solution will be prepared. (For example, 20 µl DNCB stock solution will be added to 4980 µl of THP-1 culture medium, diluting the stock (1:249 fold).) $NiSO_4$ Stock Solution: A 10 mg/ml $NiSO_4$ stock solution will be prepared. (For example, 20 mg (0.02 g) of $NiSO_4$ will be weighed in a sterile tube or volumetric flask and brought to a total volume of 2 ml with saline.) $NiSO_4$ Working Solution: A 200 µg/ml $NiSO_4$ working solution will be prepared. (For example, 100 µl $NiSO_4$ stock solution will be added to 4900 µl of THP-1 culture medium, diluting the stock (1:49 fold).)
Negative Control:	Lactic Acid (LA, final test concentration 1000 µg/ml). LA Stock Solution: A 100 mg/ml LA stock solution will be prepared. (For example, 100 mg of LA will be added to saline to a total volume of 1000 µl.) LA Working Solution: A 2000 µg/ml LA working solution will be prepared. (For example, 100 µl LA stock solution will be added to 4900 µl of THP-1 culture medium, diluting the stock (1:49 fold).)

Section 5.	CONTROLS, MEDIA, AND ASSAY SOLUTIONS (cont'd)
Medium Control:	Cell culture medium without the addition of test article or control. RPMI-10 Medium: RPMI-1640 medium (Gibco, cat. no. 22400-089, or equivalent) supplemented with 1% Penicillin-Streptomycin, 0.05 mM 2-Mercaptoethanol, and 10% fetal bovine serum. Preparation: • Dilute a 14.2 M 2-Mercaptoethanol stock solution 1:99 with tissue culture water (e.g., 10 µl stock plus 990 µl water) to yield a 0.142 M 2-Mercaptoethanol solution • Combine the following: o 432.5 ml of RPMI-1640 medium o 176 µl of the 0.142 M 2-Mercaptoethanol solution o 12.5 ml 1 M HEPES buffer solution o 5 ml Penicillin-Streptomycin o 50 ml Fetal Bovine serum • Filter-sterilize via a 0.2-µm filter Storage and Expiration: The supplemented culture medium (RPMI-10) will be stored at 2-8°C and must be used within one month. The culture medium must be warmed to room temperature just before use.
Assay Solutions:	Flow Cytometry (FACS) Buffer: A 0.1% BSA solution in dPBS will be prepared within one day of PI and antibody staining, filter-sterilized via a 0.2-µm filter, and refrigerated at 2-8°C until use. The FACS buffer may be stored no longer than two weeks.
	Blocking Solution: A 1% globulin solution (e.g., IgG_1) in dPBS will be prepared on the day before antibody staining, and refrigerated at 2-8°C until use. This globulin solution may be stored no longer than one week. Just before use, the blocking solution will be prepared by diluting the 1% globulin solution 1:1000 with FACS buffer.
	Propidium Iodide (PI) Stock Solution: A 1 mg/ml PI stock solution will be prepared (for example, 50 mg of PI will be brought up to a total volume of 50 ml with dPBS), and the solution will be filtered through a 0.2-µm filter. The stock solution will be divided into 10 ml aliquots for use. Aliquots will be stored at approximately -20°C in the dark for up to one year. Once thawed, aliquots will be stored at 2-8°C for up to three months, but not later than one year after preparation.
	PI Working Solution: The 1 mg/ml stock solution of PI will be diluted with dPBS to yield a 12.5 µg/ml PI solution, which will be stored at 2-8°C in the dark until use. The PI working solution may be stored no longer than 48 hours.

Section 6. EXPERIMENTAL DESIGN	
Reactivity Check:	The assay will first be conducted using only controls, not test articles. Only those cell cultures which pass the reactivity check will be used in the assay.
Viability Screen:	Eight concentrations of the test article will be tested. Concentrations will be prepared by diluting the stock test article solution in 1:1 serial dilutions starting from 100 mg/ml in saline, 500 mg/ml in DMSO, or the HSC. If the CV75 can be determined, the screen will be repeated to confirm the CV75 value. If the CV75 cannot be determined, or if the CV75 is very close to the limit of the concentration range, the concentration series will be adjusted (but not exceeding the maximum concentration as described above) until the CV75 can be calculated. The screen will be conducted at least twice, in independent assays. The mean CV75 will be calculated.
Main Test:	Once the mean CV75 has been determined, eight concentrations (µg/ml) will be used for the test article. These concentrations will be 1.2x CV75, and serial dilutions of $1/1.2^x$ (eight doses ranging from 0.335x CV75 to 1.2x CV75). If the CV75 cannot be determined (i.e., if sufficient cytotoxicity is not observed in the screen) the HSC will be prepared as the starting dose. The final maximum concentration of the test article is not to exceed 5000 µg/ml for saline or 1000 µg/ml for DMSO. The main test will be conducted at least twice, in independent assays.

Section 7. TEST PROCEDURE – REACTIVITY CHECK OF CELLS

The cells will be checked for reactivity at least two weeks after thawing a new cell batch

Preparation of Cell Suspension:	Cells will be centrifuged (approximately 250 xg for approximately 5 minutes at 2-8ºC) and re-suspended in fresh culture medium at $2x10^6$ cells/ml. Using a pipette, 500 µl of cell suspension will be dispensed into the wells of a 24-well tissue culture plate.
Cell Dosing:	500 µl of the working solution for each control will be added to the cell suspension in the appropriate well. The final concentrations of each control are 4 µg/ml for DNCB, 100 µg/ml for $NiSO_4$, and 1000 µg/ml for LA. The cells will be incubated for approximately 24 hours (37±1ºC, 5±1% CO_2). All dosing will be conducted under yellow light.
Cell Staining:	Following the 24-hour incubation, the cells will be stained with PI and with CD86, CD54, or isotype control antibodies using the following procedure: 1. Transfer cells from each well to 1.5 ml Eppendorf tubes 2. Centrifuge at approximately 250 xg for approximately 5 minutes at 2-8°C 3. Aspirate supernatant 4. Re-suspend cells in 1 ml cold FACS buffer 5. Repeat wash steps (steps 2-4) for a total of two washes 6. Re-suspend cells in 600 µl blocking solution and incubate at 2-8°C for approximately 15 minutes 7. Transfer a 180-µl aliquot of cell suspension (approximately 3 x 10^5 cells/well) into three wells of a round-bottom plate 8. Centrifuge at approximately 250 xg for approximately 5 minutes at 2-8°C and aspirate supernatant 9. Dilute antibodies by bringing a volume of each antibody (see below) to a total volume of 50 µl with FACS buffer and add to the appropriate wells of the plate o CD86: 6 µl (e.g., add 6 µl antibody to 44 µl FACS buffer) o FITC IgG isotype: 3 µl (e.g., add 3 µl antibody to 47 µl FACS buffer) o CD54: 3 µl (e.g., add 3 µl antibody to 47 µl FACS buffer) 10. Incubate at 2-8°C for approximately 30 minutes 11. Centrifuge at approximately 250 xg for approximately 5 minutes at 2-8°C 12. Aspirate supernatant 13. Re-suspend cells by adding 150 µl FACS buffer 14. Repeat wash steps (steps 11-13) for a total of two washes 15. Re-suspend cells in 200 µl of FACS buffer and transfer to flow cytometry tubes 16. Add 10 µl of PI solution to each flow cytometry tube to obtain a final PI concentration of 0.625 µg/ml 17. Analyze by flow cytometry

Section 7.	**TEST PROCEDURE – REACTIVITY CHECK OF CELLS (cont'd)**
Flow Cytometry:	Analyses will be performed with a flow cytometer using 15 mW of power at 488 nm excitation wavelength. BD CellQuest™ ver. 3.3 acquisition software on a Macintosh G4 acquisition system will be used to capture and store data on a dedicated network drive. Data files will be analyzed using CellQuest™ to determine appropriate analysis gates.
Cell Viability:	Cell viability will be measured by flow cytometry, gating out dead cells stained with PI. A total of 10,000 living cells will be acquired (when viability is low, up to 30,000 cells, including dead cells, will be acquired). Viability will be calculated as percent of total cells by the following equation: $$\text{Cell Viability} = \frac{\text{Number of living cells}}{\text{Total number of acquired cells}} \times 100$$
Reactivity Check Acceptance:	The reactivity check will be considered acceptable if: • The viability of non-treated cells cultured in the culture medium for 24 hours is more than 90% • Treatment of the cells with DNCB and $NiSO_4$ produces a positive response for both CD86 and CD54 expression • Treatment of the cells with LA produces a negative response for both CD86 and CD54 expression
Repeating the Reactivity Check:	If the results of the reactivity check fail the acceptance criteria, the cells will be cultured for one more passage and the reactivity check will be repeated. If the second reactivity check again fails the acceptance criteria, a new lot of cells will be thawed, cultured for two weeks, and the reactivity check will be conducted.

Section 8. TEST PROCEDURE – SCREEN

The screen will be conducted at least twice, in independent assays. If the results of the second (confirmatory) screen are not consistent with those of the first screen, a third screen will be conducted.

Preparation of Cell Suspension:	Will be performed in the same manner as for the reactivity check (Section 7).
Cell Dosing:	A volume of 500 µl of the working solution for each test article concentration and control will be added to the cell suspension in the appropriate well. The cells will be incubated for approximately 24 hours (37±1ºC, 5±1% CO_2). Care should be taken to avoid evaporation of volatile test articles and cross-contamination between wells by test articles (for example, by sealing the plate with Parafilm® prior to incubation).
Cell Staining:	Following the 24-hour incubation, the cells will be stained with PI using the following procedure: 1. Remove cells from each well and transfer them to flow cytometry tubes 2. Centrifuge the cells at approximately 250 xg for approximately 5 minutes at 2-8°C 3. Aspirate supernatant and re-suspend the cells in 600 µl of FACS buffer 4. Transfer 200 µl of the cell suspension to a 96-well round-bottom or V-bottom plate 5. Centrifuge the cells in the 96-well plate at approximately 250 xg for approximately 5 minutes at 2-8°C 6. Wash cells twice with 200 µl FACS buffer 7. After the second wash, re-suspend the cells in 200 µl of FACS buffer 8. Just before flow cytometry analysis, add 10 µl of PI 9. Transfer cell suspensions to flow cytometry tubes and analyze by flow cytometry
Estimation of CV75 Value:	The CV75 value for each screen will be derived from the dose response curve and the following equation: $$\textbf{Log CV75} = \frac{\textbf{(75-C) x Log B – (75-A) x Log D}}{\textbf{A – C}}$$ Where: A = viability of concentration above cell viability of 75% B = concentration of that data point A C = viability of concentration below cell viability of 75% D = concentration of data point C

Section 9. TEST PROCEDURE – MAIN TEST

The main test will be conducted at least twice, in independent assays. If the results of the second (confirmatory) main test are not consistent with those of the first main test, a third main test will be conducted. The main test will be conducted in the same manner as in the reactivity check (Section 7).

Cell Viability:	Cell viability will be measured by flow cytometry, gating-out dead cells stained with PI. A total of 10,000 living cells will be acquired (when viability is low, up to 30,000 cells, including dead cells, will be acquired). The Mean Fluorescence Intensity (MFI) of the viable cells and the viability of each sample will be obtained.
Quality Checks of Assay:	Positive Controls: • DNCB and $NiSO_4$ should each produce a positive response for CD86 (RFI ≥150) and CD54 (RFI ≥200) vs. the negative control. • Cell viability should be more than 50%.
	Vehicle Control: • Cell viability of medium and DMSO controls should be more than 90%. • DMSO RFI values compared to medium control for both CD86 and CD54 should not exceed the positive criteria (CD86 ≥150 and CD54 ≥200) vs. the media control. • For both medium and DMSO controls, the MFI ratio for both CD86 and CD54 to isotype control should be more than 105%.
	Test Article: • The cell viability of at least four test article concentrations in each assay should be 50% or more. • Negative results are acceptable only for test chemicals exhibiting cell viability at 1.2x CV75 of less than 90%. Negative results with cell viability of 90% or higher are discarded. The screen should be repeated to determine the CV75 determination. • Positive results for test articles of any cell viability at 1.2x CV75 are acceptable. • It should be noted that when 5000 µg/ml in saline, 1000 µg/ml in DMSO, or the highest soluble concentration is used as the maximal test concentration of a test chemical, the results are acceptable.

Section 10. DATA ANALYSIS

MFI:	The Geometric Mean (GeoMean) Fluorescence Intensity (MFI) for each well will be measured by flow cytometry.
RFI:	The Relative Fluorescence Intensity (RFI) will be used as an indicator of CD86 and CD54 expression, and will be calculated as follows for each concentration of every chemical: $\text{RFI} = \frac{\text{MFI of chemical-treated cells – MFI of chemical-treated isotype cells}}{\text{MFI of solvent treated cells – MFI of solvent-treated isotype cells}}$ When corresponding cell viability is less than 50%, the Relative Fluorescence Intensity (RFI) will not be used, due to cytoplasmic debris.
Calculation of EC150 and EC200:	When test article concentrations yield RFI values both above and below the positive criteria (RFI = 150 for CD86, and RFI = 200 for CD54), the effective concentration (EC) values (i.e., the concentration at which the test article induced an RFI of 150 or 200) will be calculated according to the following equations: **EC150 (for CD86)** = $B_{dose} + [(150 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$ **EC200 (for CD54)** = $B_{dose} + [(200 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$ Where: A_{dose} is the lowest concentration in µg/ml with RFI > 150 (CD86) or 200 (CD54) B_{dose} is the highest concentration in µg/ml with RFI < 150 (CD86) or 200 (CD54) A_{RFI} is the RFI at the lowest concentration with RFI > 150 (CD86) or 200 (CD54) B_{RFI} is the RFI at the highest concentration with RFI < 150 (CD86) or 200 (CD54)
Prediction Model:	If the RFI of CD86 is equal to or greater than 150 at any test dose (≥50% of cell viability) in at least two independent assays, AND/OR if the RFI of CD54 is equal to or greater than 200 at any tested dose (≥50% of cell viability) in at least two independent assays, the chemical prediction will be considered positive. Otherwise it will be considered negative. If the first two independent assays are not concordant, a third assay must be performed and the final prediction will be based on the mode of the conclusion from the three individual assays (i.e., two out of three.)
Kow Note:	Test articles with Log Kow[1] (calculated using KOWWIN™, SPARC or ALOGPS) of up to 3.5 have been tested successfully. However, test articles with a Log Kow of greater than 3.5 may still be tested at lower soluble concentrations. In such a case, a negative result should be considered inconclusive, whereas a positive result could still be used to support the identification of test article as a skin sensitizer. Up to six assays, meeting the requirements for qualified testing are permitted to reach a conclusion for a test article.

[1]The octanol/water partition coefficient (Kow) is defined as the ratio of a chemical's concentration in the octanol phase to its concentration in the aqueous phase of a two-phase octanol/water system.

Section 11. TEST DURATION

Duration:	The duration of the Human Cell Line Activation Test will be approximately two to four weeks – including a reactivity check, at least two independent assays of CV75 determination and at least two assays for CD86 and CD54 determination.

Section 12. REFERENCES

1. Takenouchi, O., Miyazawa, M., Saito, K., Ashikaga, T., and Sakaguchi, H. Predictive performance of the human Cell Line Activation Test (h-CLAT) for lipophilic chemicals with octanol-water partition coefficients. The Journal of Toxicological Sciences (J. Toxicol, Sci,) Vol 38, No.4, 599-609, 2013.

2. EURL ECVAM DataBase service on Alternative Methods to animal experimentation (DB-ALM) protocol no. 158: Human cell line activation test (h-CLAT), 2014.

3. Draft OECD Guideline "*In Vitro* Skin Sensitisation: Human Cell Line Activation Test (h-CLAT).

4. KOWWIN™, in Estimation Program Interface (EPI) suite™, Environmental Protection Agency, Washington, DC, USA (http://www2.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface)

5. SPARC, ARChem (http://archemcalc.com/sparc/)

6. ALOGPS, Virtual Computational Chemistry Laboratory (http://vcclab.org/lab/alogps/)

Section 13. PROTOCOL REVISIONS

Revisions:	Any amendment to or deviation from this protocol will be fully documented in the study file, including the reason for the change, the authority for said change and the date.

Section 14. RECORDS TO BE MAINTAINED	
Collection of Data:	All data generated during the conduct of this study will be recorded in ink on data collection forms. All entries will be dated, initialed and verified per MB Standard Operating Procedures (SOPs).
Final Report:	The final report will include, but is not limited to, a description of the methods and experimental design, results, discussion, conclusion, data tables and the Quality Assurance statement. The content of the final report will meet the requirements of the applicable Good Laboratory Practice Regulations.
Retention of Data:	All data generated during the conduct of this study will be archived at MB Research for at least 10 years from the date of the final report. The Sponsor will be contacted in writing to determine final disposition of the records. If the Sponsor fails to respond within 90 days, the archived items will be properly discarded.
Raw Data:	Raw data will be filed at MB Research by project number.
Final Reports:	The final report will be filed at MB Research by Sponsor name and MB project number.
Test Article:	Refer to the Sponsor Request section for test article disposition. If this study exceeds 28 days, it is recommended that the Sponsor archive a sample of the test article to meet the applicable Good Laboratory Practices Regulations.
Test Article Mixtures:	These are not routinely retained. However, upon written request from the Sponsor, an aliquot of the test article mixture will be forwarded to the Sponsor (at an additional cost).

Section 15. GOOD LABORATORY PRACTICES	
This study will be conducted in accordance with the current Good Laboratory Practice Regulations of the EPA, 40 CFR Part 160 and 792, the FDA, 21 CFR Part 58,and OECD, Principles on Good Laboratory Practice.	
Protocol:	MB Research will have on file a copy of this protocol, signed and dated by the responsible MB Study Director and dated by the Sponsor Representative.
Quality Assurance:	The Quality Assurance Unit will inspect at least one critical phase of this study, audit the raw data and audit the report in accordance with the protocol, MB Research SOPs and applicable regulatory requirements.

Section 16. SPONSOR REQUEST		
Protocol:	Implement as written	
Report Submission:	Yes, to the EPA	
Test Article Identity:	*(The identity that will be used in the **report** and supporting documentation):* JA900-DAA (Lot PT-917-59)	
Characterization:	Yes, test article characterization is provided.	*(if provided)*Performed according to NEITHER GLP or GMP
Storage Requirements:	Room temperature Protect from light	
Additional Information:	The test material is a polymer in ethanol solution, concentration 51%	
Disposition:	Discard at study termination - no charge	*If test article is returned to a address different from that below, please specify:*
Authorization Statement:	This protocol is authorized for implementation at MB Research.	
Confidentiality:	Study results and reports will be released only to the below-named Sponsor Representative unless additional Sponsor Representatives are identified below.	
Authorization Date:	05Jul2016	
Sponsor Name:	Xiao Huang	**Title:**Regulatory Manager
Email Address:	Xiao.huang@iff.com	**Phone:** 732-203-8136
Company Name:	IFF	**Address:**800 Rose Lane, Union Beach NJ 07735
Additional Sponsor Representative(s):	**Name(s):**	**Email Address:**

Section 17. MB RESEARCH ACKNOWLEDGEMENT

Request for implementation of this protocol and receipt of the test article is acknowledged by MB Research.

Test Article Identity:	JA900-DAA (Lot PT-917-59)		
Characterization:	Yes, test article characterization was provided. *(if provided)*	☐Incomplete characterization was provided ☑Complete characterization was provided	
MB Project #:	16-24502.41		
Supplier:	The cell line supplier is:American Type Culture Collection (ATCC)		
Proposed Experimental Start Date:	11JUL16	**Proposed Experimental Termination Date:**	11 OCT 16
Completion Date:	The report will be submitted approximately six weeks following the experimental termination date.		
Approval:	This protocol is approved for implementation at MB Research by the below-named MB Study Director.		
Approved By:	Michael DeFe **Date:** 07JUL16 **Time:** 1103		

Study Director
Testing Facility: MB Research Laboratories
1765 Wentz Road, P. O. Box 178
Spinnerstown, PA 18968

	MB Research Protocol Amendment	Page 1 of 1
Study Title:	Human Cell Line Activation Test (h-CLAT	
Project No.:	16-24502.41	**Protocol ID:** 705

Amendment No.:	1
Statement of Amendment:	Solubility will not be conducted and based-off previous knowledge of material's solubility. Screen will be conducted starting at a top dose of 5000 ug/ml.
Reason:	Sponsor's Request.
Effective Date:	12 Jul 2016

Study Director:		18OCT16
	Signature	**Date**

Select One (✓)	**Sponsor Acknowledgement**
	Sponsor **requested** amendment via email dated: _____, maintained in the study file.
	Sponsor **requested** amendment via phone conversation dated: _____, maintained in the study file.
✓	Sponsor **notified** of amendment via email dated: 12 Jul 2016 _____, maintained in the study file.
	Sponsor **notified** of amendment via phone dated: _____, maintained in the study file.

Select ✓ or NA	**Institutional Animal Care and Use Committee Approval**
NA	If applicable, IACUC **approved** amendment on:_____, documentation maintained in the study file.